Stable Isotopes in Ecology and Environmental Science

SECOND EDITION

EDITED BY
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STABLE ISOTOPES IN ECOLOGY AND ENVIRONMENTAL SCIENCE

CHAPTER 14

Compound-specific stable isotope analysis in ecology and paleoecology

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Introduction

Compound-specific stable isotope ratio mass spectrometry enables the molecular specificity and isotopic signature of compounds to be exploited concomitantly, to provide a powerful tool for tracing the origin and fate of organic matter in both extant and fossil ecosystems. The scope of applications is enormous, with the pollution, paleoenvironmental and archeological fields being examples of the increasing number of subject areas benefiting from the use of compound-specific approaches. Indeed, as will be shown below such approaches can be used to investigate chemical and biological processes and metabolism in both whole ecosystems (marine, terrestrial, freshwater) and individual organisms of all classes, i.e. microbes to mammals.

Even before the advent of new on-line technologies, the stable isotopic compositions of individual compounds had begun to be rigorously investigated, e.g. Abelson & Hoering (1961). Such studies relied upon classic purification methods and required relatively large sample sizes. The results of such investigations underpin much of our biochemical understanding of interpretations of bulk stable isotope determinations and certainly provided the impetus for the development of improved instrumental approaches (Galimov 1981). The introduction of high performance liquid chromatography provided an effective means of isolating milligram quantities of highly purified compounds of widely varying polarity for off-line combustion and stable isotope analysis. The major breakthrough in compound-specific stable isotope analysis came with the commercial production of the gas chromatograph/combustion/isotope ratio mass spectrometer (GC/C/IRMS) in the early 1990s. The technical aspects of such instruments are discussed in detail below. Their availability resulted in an explosion of compound-specific stable isotope analyses, particularly amongst the organic geochemical community, for the purposes of paleoenvironmental reconstruction (Merritt et al. 1994). However, opportunities rapidly began to be recognized for the application of GC/C/IRMS by members of other scientific communities, including ecologists (Meier-Augenstein 1999; Boschker & Middelburg 2002).

Why use compound-specific stable isotopes?

There are a number of compelling reasons for employing compound-specific stable isotope approaches (rather than more traditional bulk measurements), which derive from the fact that:

1 Different biochemical components, even those within a single organism, can possess different stable isotope values, e.g. $\delta^{13}C$ values of carbohydrates and lipids in plants, and isoprenoids vs. aliphatic lipids.
2 Structurally similar biochemical components of ecological materials can derive from a range of sources potentially exhibiting different stable isotopic signatures, e.g. palmitic acid in soil can derive from plants, invertebrates, or microbes.
3 Biogenic organic matter, either living or dead, is chemically complex such that changes in the bulk stable isotope value may occur as a result of changes in chemical composition, i.e. losses or gains of components with stable isotope values that differ from the bulk (mean) stable isotope value may be erroneously interpreted as fractionation phenomena, e.g. preferential degradation of isotopically heavy components would lead to an overall lowering of bulk stable isotope values.
4 Stable isotopic analysis of individual components in chemically complex materials, e.g. sedimentary biomarkers, can reveal characteristic stable isotopic signatures of contributors mediating processes that would otherwise be masked in bulk stable isotope values.
5 The complementary use of structurally diagnostic biomarkers together with their compound-specific stable isotope values provides biological process or biochemical pathway-based information inaccessible through bulk stable isotope analyses.
6 Different biochemical components, even identical components within different pools, of a living organism can possess significantly different turnover times that would be undetectable by bulk analysis and may even lead to erroneous interpretations of trends seen in recorded stable isotope values, especially in temporal studies, e.g. essential and nonessential components or structural vs. storage substances.
7 Where genuine kinetic isotope-based fraction effects exist their source can only be determined through stable isotopic assessments at the level of the biochemical component and specific pathway.

It is therefore essential that in order to use light stable isotopes effectively in ecological research a mechanistic understanding of the biochemical factors that underpin stable isotope signals be developed. This principle applies no matter what stable isotope or combination of isotopes is being employed or what ecosystem is under investigation. Unless we strive for a level of understanding that links biochemistry to stable isotope composition it is unlikely that we can ever rigorously interpret the results of bulk stable isotopes or exploit the use of light stable isotopes in ecological research to their fullest potential.

Compound-specific stable isotope analyses are more complex to undertake than bulk stable isotope analyses and require that careful consideration be given to sample preparation protocols. Such factors that need to be considered are:

1 What are the target compound(s)?
2 Whether there is a possibility of compounds existing in more than one physical or chemical state within a complex environmental or biological matrix?
3 Whether or not the target compounds are amenable to gas chromatography (GC) analysis directly or will require derivatization?
4 Which is the most suitable derivatizing agent for GC/C/IRMS analysis; this is not necessarily the most appropriate derivative if only GC analysis was intended (the specific considerations to GC derivative selection are given later)?
5 What is the most suitable GC column (stationary phase and column dimensions)?
6 Finally, and probably most important of all, are the target compounds fully separable from other eluting compounds, i.e. can **baseline resolution** be achieved?

Each of these factors will be dealt with in the following sections.

Analytical considerations in compound-specific stable isotope analysis

The overall aim of any compound-specific stable isotope analysis is to provide accurate stable isotope value(s) for a specific component(s) of what is likely to be a biochemically complex matrix containing many tens, hundreds or even thousands of components of widely varying chemical and physical states. Thus, the aim will be to purify that compound avoiding, or accounting for, protocol-induced isotope effects such that the recorded stable isotope value will be comparable, within reasonable analytical error, to that of the compound in its natural state. Given that the primary means of obtaining compound-specific stable isotope values will be via GC/C/IRMS, then sample preparation schemes will comprise the following steps: (i) extraction, (ii) separation, and (iii) derivatization (in the case of functionalized compounds). General and specific features of such schemes are presented in turn below.

Sample preparation

The majority of the practical aspects of sample preparation have been covered by Teece & Fogel (2004), hence, only a brief summary will be

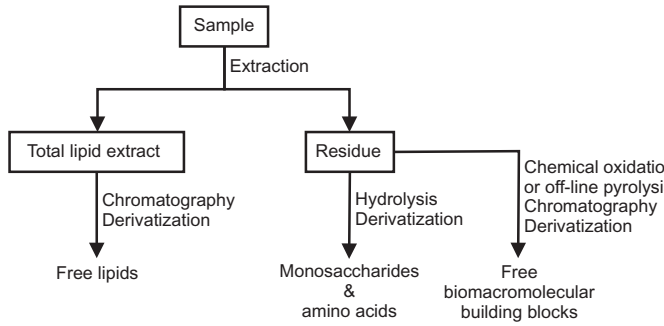


Figure 14.1 A schematic overview of an isolation procedure.

given here. Figure 14.1 shows a typical protocol for the isolation of the most common compounds targeted for compound-specific stable isotope analysis. Before extraction, samples are dried and crushed to ensure homogeneity and increase the effectiveness of solvent penetrating the sample matrix. Methods available for lipid extraction include: Soxhlet (large samples; tens to hundreds of grams; heat stable), ultrasonication (small samples, <10 g) and liquid/liquid extraction. The Bligh-Dyer method is specifically designed for the extraction of fresh biological tissues (Bligh & Dyer 1959; Smedes & Askland 1999; Manirakiza et al. 2001). No matter which extraction method is employed the resulting total lipid extract (TLE) is then further separated into different compound classes using various chromatographic methods (Kim & Salem 1990; Touchstone 1991, 1993, 1995; Alvarez & Touchstone 1992; Myher & Kuksis 1995; Abidi, 2001). Occasionally, a single chromatographic technique does not yield baseline-resolved peaks in the GC/C/IRMS analysis and a combination of techniques is required. Whichever technique(s) is selected it is important to verify whether or not aspects of the analytical protocol will introduce an isotopic fractionation effect by analysis of reference compounds of known stable isotopic composition.

Many important components of ecological materials cannot be analyzed directly by GC. For example, complex lipids require chemical cleavage to yield GC amenable components, i.e. commonly occurring phospholipids are saponified and methylated to generate fatty acid methyl esters (FAMEs; Crossman et al. 2004). Likewise, solvent unextractable biopolymers such as carbohydrates, proteins, lignin, or aliphatic biomacromolecules, e.g. suberin or cutin, must be chemically, enzymatically, or pyrolytically cleaved (Hedges & Ertel 1982; Poole & van Bergen 2002; Anwar et al. 2004; Poole et al. 2004; Stefanova et al. 2004). Care must be taken to ensure that the cleavage reactions are complete, since incomplete reactions may result in kinetic isotope effects (Jim et al. 2003a).

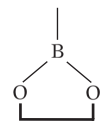
Derivatizations for compound-specific stable isotope analysis

GC/C/IRMS instrumentation enables the compound-specific isotope analysis of individual organic compounds, for example, *n*-alkanes, fatty acids, sterols, amino acids, and monosaccharides, extracted and purified from bulk organic materials. The principle caveat of compound-specific work is the requirement for chemical modification, or derivatization, of compounds containing polar functional groups primarily to enhance their volatility prior to introduction to the GC/C/IRMS. Table 14.1 summarizes the most commonly employed procedures for derivatization of polar, nonvolatile compounds for compound-specific stable isotope analysis using GC/C/IRMS.

The addition of a derivative group (or several derivative groups in the case of polyfunctionalized compounds, such as amino acids and monosaccharides) introduces exogenous carbon which ultimately alters the $\delta^{13}C$ value of the compound of interest. Derivatization of fatty acids and sterols, to FAMEs and sterol trimethylsilyl ethers, respectively, is relatively straightforward since the resulting sample-to-derivative carbon molar ratio is high, resulting in minimal analytical error. Additionally, derivatization reactions, such as esterification and silylation are typically rapid and quantitative thereby precluding kinetic isotope effects (Rieley 1994).

In contrast, derivatization of small, polyfunctional compounds, such as amino acids and monosaccharides presents a more complex analytical challenge. The most prevalent procedures for derivatization of amino acids for GC analysis involve either silylation, to *tert*-butyldimethylsilyl (tBDMS) or trimethylsilyl (TMS) derivatives or a multi-step procedure, involving esterification of the carboxylic acid group with an acidified alcohol and acylation of amino, hydroxyl, and thiol groups with an anhydride, to form N(O,S)-acyl alkyl esters (Demmelmair & Schmidt 1993; Metges et al. 1996; Macko et al. 1997; Metges & Daenzer 2000; Docherty et al. 2001; O'Brien et al. 2002; Jim et al. 2003a; Corr et al. 2005). While trialkylsilylation represents a rapid, one-step reaction, trialkylsilylated derivatives possess relatively poor stability and the addition of several carbon atoms in the derivative alters the $\delta^{13}C$ value of the compound of interest, which results in considerable analytical error, especially in small molecules (Derrien et al. 2003). Also, the requirement of baseline resolution, a prerequisite for obtaining reliable isotope measurements, is rarely achieved using silylated derivatives in these cases due to their apolar character. Derivatization of amino acids and monosaccharides with TMS groups can involve the formation of several products of one compound (i.e., mono-, di-, and tri- TMS, and isomeric derivatives; Evershed 1993; Meier-Augenstein 1997). Finally, it has been postulated that silicon in *t*BDMS groups forms silicon carbide (SiC_x) in the oxidation reactor of the GC/C/IRMS, resulting in incomplete analyte combustion to CO_2 (Shinebarger et al. 2002; Derrien et al. 2003). However, after nearly 10 years

Table 14.1 Summary of the most commonly employed procedures for derivatization of polar, nonvolatile compounds for compound-specific stable isotope analysis using GC/C/IRMS.

Procedure	Functional group	Mechanism	Reagent	Product	References
Silylation (sterols, alcohols, amino acids, monosaccharides, lignin phenols)	–OH –CO_2H –NH_2 –NHR –SH	*tert*-butyldimethyl-silylation (tBDMS)	*N-tert*-butyldimethylsilyl-*N*-methyl-trifluoroacetamide (MTBSTFA)	O/N/S-TMS	Lockheart et al. (1997) Stott & Evershed (1996) Derrien et al. (2003)
		Trimethylsilylation (TMS)	*N,O*-bis(trimetylsilyl) trifluoro-acetamide (BSTFA)	O/N/S-tBDMS	
Esterification (fatty acids, amino acids)	–CO_2H	Methylation	BF_3/methanol Acetyl chloride/methanol	CO_2CH_3	Howland et al. (2003) Docherty et al. (2001)
		n/2-propylation	Acetyl chloride/ *n/2*-propanol	$CO_2(CH_2)_2CH_3$ $CO_2CH(CH_3)_2$	
Acylation (amino acids, monosaccharides)	–OH –NH_2 –NHR –SH	Acetylation	Acetic anhydride	O/N/S–C=OCH_3	Demmelmair & Schmidt (1993) Docherty et al. (2001)
		Trifluoroacetylation	Trifluoroacetic anhydride	O/N/S–C=OCF_3	Metges & Daenzer (2000)
		Pivaloylation	Pivaloyl chloride	O/N/S–C=$OC(CH_3)_3$	
Methylboronatrion (MBA) (monosaccharides)	–OH	Cyclization of two adjacent –OH groups	Methane boronic acid (MBA)		Van Dongen et al. (2001)

of undertaking GC/C/IRMS analyses of cholesterol as its TMS ether, no undue deterioration of instrument performance has ever been observed (Stott & Evershed 1996; Jim 2000).

The alternative approach, acylation, represents a widely employed method of derivatizing hydroxyl groups of amino acids and monosaccharides, despite the kinetic isotope effect (KIE) associated with the procedure, which precludes the direct calculation of compound-specific $\delta^{13}C$ values via a simple mass balance equation. However, because such reactions have been shown to be reproducible for each amino acid and monosaccharide, the KIE can be accounted for by employing empirical correction factors ($\delta^{13}C_{corr}$) to calculate the "effective" $\delta^{13}C$ value of the reagent where fractionation occurs (Demmelmair & Schmidt 1993; Rieley 1994; Docherty et al. 2001). Hence, three acylation procedures, in combination with esterification, are now routinely employed for amino acid $\delta^{13}C$ measurements: acetylation (+2C; Demmelmair & Schmidt 1993; Metges et al. 1996), trifluoroacetylation (+2C; Macko et al. 1997; Docherty et al. 2001; O'Brien et al. 2002; Howland et al. 2003; Jim et al. 2003a, 2006) and pivaloylation (+5C; Metges et al. 1996; Metges & Petzke 1997; Metges & Daenzer 2000), each advantageous over TMS (+3C) and *t*BDMS (+6C) esters because of their overall lower carbon addition to each amino acid. *N*-trifluoroacetyl iso-propyl (*N*TFA-IP) esters are extensively employed in the stable carbon isotope analysis of amino acids due to their excellent chromatographic properties compared with *N*-acetyl iso-propyl (*N*AIP) esters. However, their compatibility with GC/C/IRMS is now uncertain because combustion of fluorinated derivatives irreversibly poisons the oxidation reactor, forming extremely stable CuF_2 and NiF_2 products with associated reduction in oxidation efficiency (Meier-Augenstein 1997). This analytical challenge has, nevertheless, been resolved by analysing amino acid *N*AIP esters on a high-polarity stationary phase to prevent the previously reported poor chromatography and co-elution problems identified for these derivatives on the traditionally employed non-/low-polarity phases.

Existing acetylation techniques for monosaccharides have also been adapted for determining $\delta^{13}C$ values of these compounds, of which the most common is the formation of alditol acetates via a two step-reaction, comprising their reduction with sodium borohydride, followed by acetylation of all alcohol groups with acetic anhydride using either pyridine (Moers et al. 1993; Macko et al. 1998) or *N*-methylimidazole (Blakeney et al. 1983; Docherty et al. 2001) as a catalyst. A rigorous approach to error propagation is essential in the use of these derivatives to combine both the imprecision associated with the use of correction factors to account for the KIE and also the high derivative-to-sample carbon molar ratio of alditol acetates (Rieley 1994; Docherty et al. 2001).

Van Dongen et al. (2001) avoided the KIE associated with acetylation reactions in their derivatization of monosaccharides for carbon isotope work,

instead reacting analytes containing two or more adjacent hydroxyl groups with methylboronic acid, followed by trimethylsilylation of the remaining hydroxyl groups using *N,O*-bis(trimethylsiyl)trifluoroacetamide (BSTFA). While this method also involves lower carbon addition (+2/3C) compared with alditol acetate derivatization (+10/12C), reduced yields have been observed for several monosaccharides indicating an incomplete reaction with associated isotopic fractionations (van Dongen et al. 2001). It was subsequently postulated that TMS groups could potentially substitute methylboronic acid on hydroxyl groups, generating by-products (Derrien et al. 2003; Gross & Glaser 2004).

Instrumentation related aspects of compound-specific stable isotope determinations

Determining the $\delta^{13}C$ and $\delta^{15}N$ values of individual compounds

GC/C/IRMS was first demonstrated by Matthews & Hayes (1978). However, it was somewhat later that Barrie et al. (1984) coupled a GC, via a combustion interface, to a dual collector mass spectrometer to produce the forerunner of today's GC/C/IRMS instruments. Even so, true determinations of $\delta^{15}N$ values of individual compounds by GC/C/IRMS remained elusive until finally demonstrated by Merritt et al. (1991). More recently the precision of GC/C/IRMS instruments has been improved further with uncertainties in $\delta^{13}C$ values as low as ±0.5‰ for samples containing 5 pmol C and ±0.1‰ for 100 pmol analytes having been demonstrated (Merritt & Hayes 1994). Instruments available commercially today all conform to the same general principals of design.

Figure 14.2a depicts a generalized schematic of a GC/C/IRMS instrument configured for determinations of $\delta^{13}C$ (or $\delta^{15}N$) values of individual compounds. Briefly, mixtures of compounds are separated by high resolution capillary GC then individually combusted online over a catalyst (CuO/Pt, 850°C or CuO/NiO/Pt, 940°C; Merritt et al. 1995) generating CO_2 and H_2O. For the determination of $\delta^{15}N$ values or $\delta^{13}C$ values of compounds containing nitrogen (N_2O is generated and causes isobaric interference with *m/z* 44 and 45; Metges & Daenzer 2000) the effluent is then passed through a second reactor where nitrogen oxides are catalytically (Cu, 600°C) reduced to N_2 (Brand et al. 1994). H_2O is removed by a water separator, typically comprising a length of water permeable Nafion™ tubing, thereby avoiding the formation of HCO_2^+ ions that would otherwise result in isobaric interference with $^{13}CO_2$ (Leckrone & Hayes 1998) it should be noted that the efficiency of this process is temperature dependent (Leckrone & Hayes 1997). For determination of $\delta^{13}C$ values the remaining CO_2 is introduced into a MS equipped with a triple collector comprising three Faraday cups monitoring simultaneously *m/z* 44, 45 and 46 corresponding to the ions of the

three isotopomers $^{12}C^{16}O_2$, $^{13}C^{16}O_2$ and $^{12}C^{18}O^{16}O$, respectively. For the determination of $\delta^{15}N$ values, the eluting CO_2 is retained in a cryogenic trap to avoid the production of isobaric CO^+ resulting from the unimolecular decomposition of CO_2^+ ions (Brand et al. 1994). Remaining N_2 is then introduced in an identical manner but this time monitoring simultaneously *m/z* 28 and 29 using the first two Faraday cups corresponding to the isotopomers $^{14}N^{14}N$ and $^{15}N^{14}N$. The resultant output currents are then amplified, digitized, and recorded by computer, which then integrates each signal and calculates the corresponding stable carbon or nitrogen isotope ratio, represented by the signal, relative to either co-injected standards or a gas standard, returning it as a δ value (Merrit et al. 1994; Ricci et al. 1994). As with any analytical technique a number of methods have been developed, ostensibly by the user base, to maximise the accuracy and precision of determinations.

The single most important requirement in performing a valid and robust determination of the $\delta^{13}C$ or $\delta^{15}N$ value of an individual compound by GC/C/IRMS is good chromatographic separation of the target compound(s) as shown in Figure 14.3, achieved by optimization of the GC operating conditions and judicious column selection. Peaks that are not fully resolved (up to 25% co-elution) can still be integrated separately as long as a minimum estimate of analytical error is gained by running the sample at different concentrations (Ricci et al. 1994). For any larger overlap, co-eluting components must be integrated as one peak using the integration software, although the errors associated with such determinations may be substantial. Another consideration when analysing isotopically enriched compounds is the possibility of carryover effects between analytical runs, i.e residual amounts of an isotopically enriched component from a previous analysis can affect the δ value subsequently determined for the same compound from a different experiment (Mottram & Evershed 2003). In resolving this problem points in the flowpath of the GC prone to the accumulation of such residues, e.g. injector liners and metal connectors, should be adequately heated and, when necessary, changed to avoid the build-up of isotopically enriched contaminants; checks with standards to assess such effects should be made at regular intervals.

In addition to the reference gas calibrant it is important that the performance of the GC/C/IRMS instrument be constantly monitored using a suite of compounds of known relative stable isotopic composition. Such references should ideally belong to the same compound class as the target compounds since the performance of the instrument for one particular class of compounds will not necessarily replicate that of a different class of compounds (Meier-Augenstein et al. 1996). In addition, a range of homologues should be utilized as a standard mixture thereby assessing the performance of the instrument across the entire temperature range utilized by the GC. By doing this factors such as leaks and/or blockages at varying temperatures can be quickly identified and resolved.

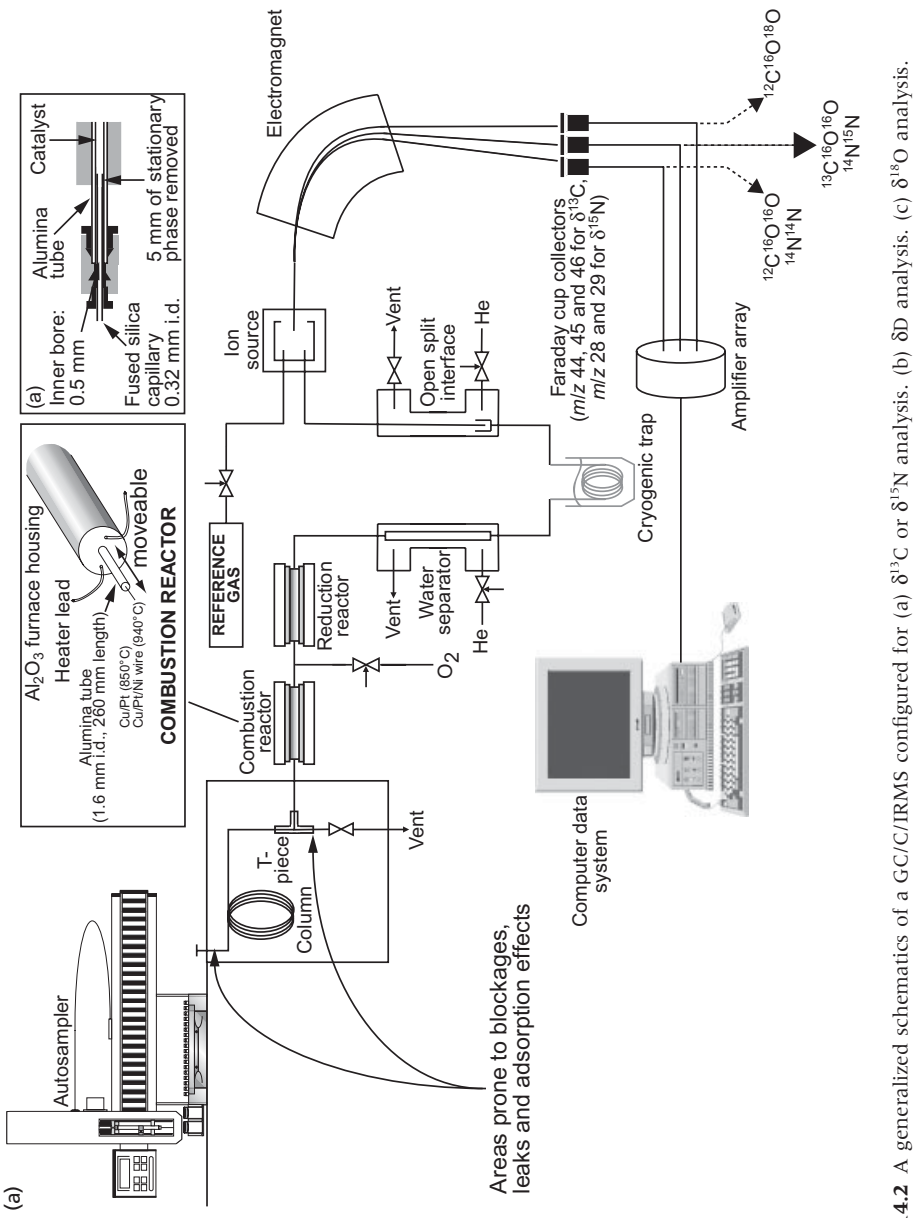


Figure 14.2 A generalized schematics of a GC/C/IRMS configured for (a) $\delta^{13}C$ or $\delta^{15}N$ analysis. (b) δD analysis. (c) $\delta^{18}O$ analysis.

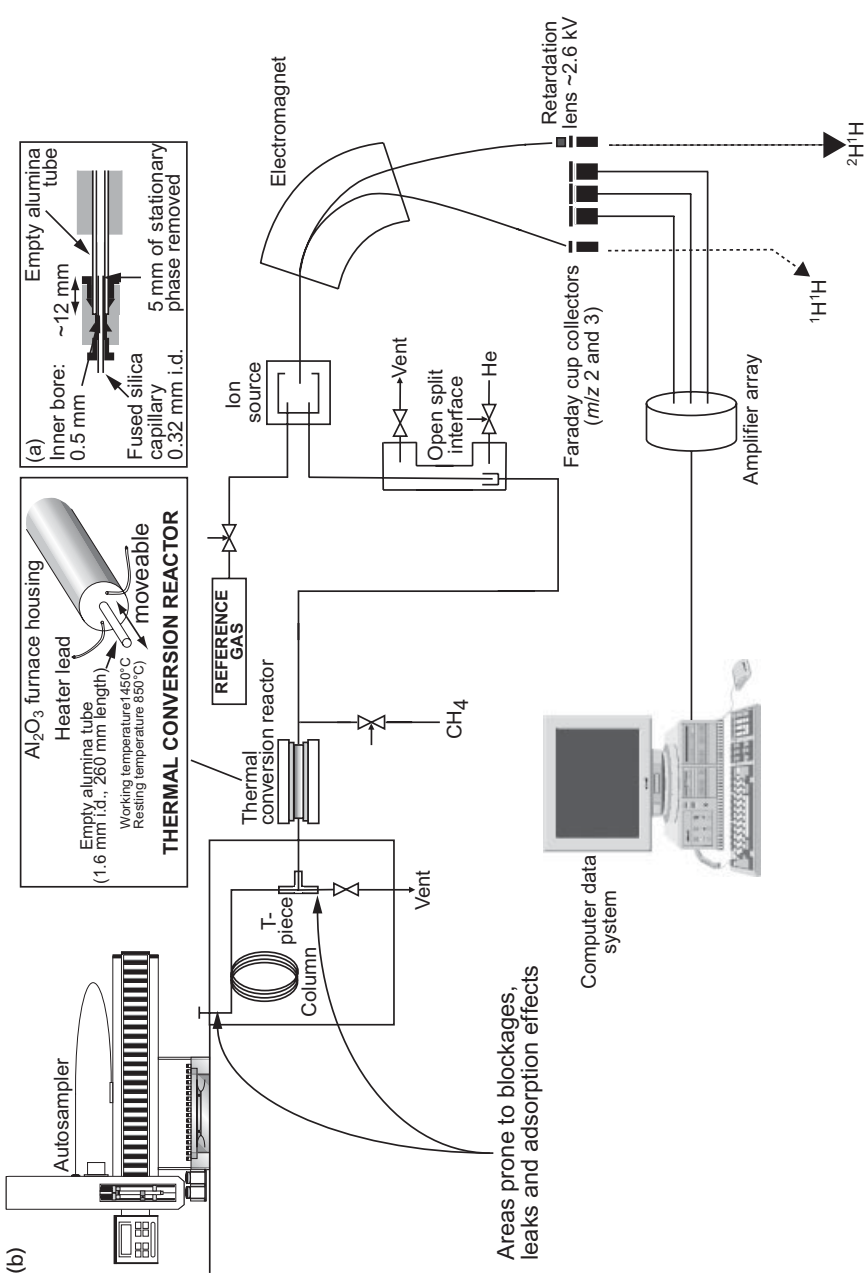


Figure 14.2 *(Continued)*

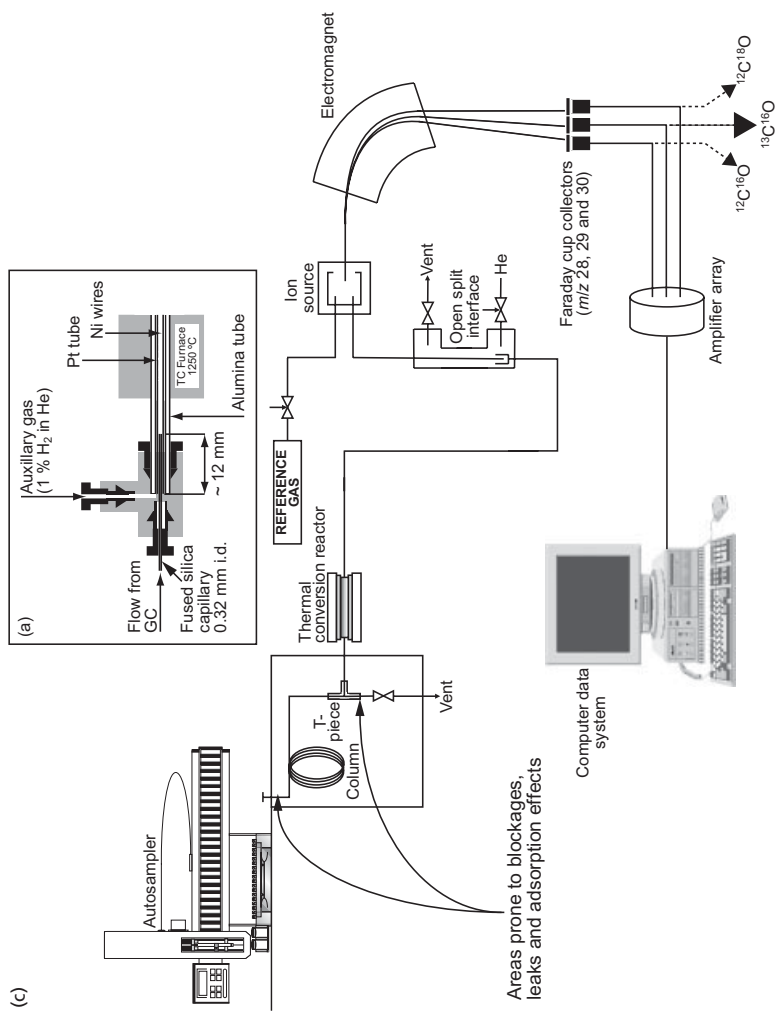


Figure 14.2 *(Continued)*

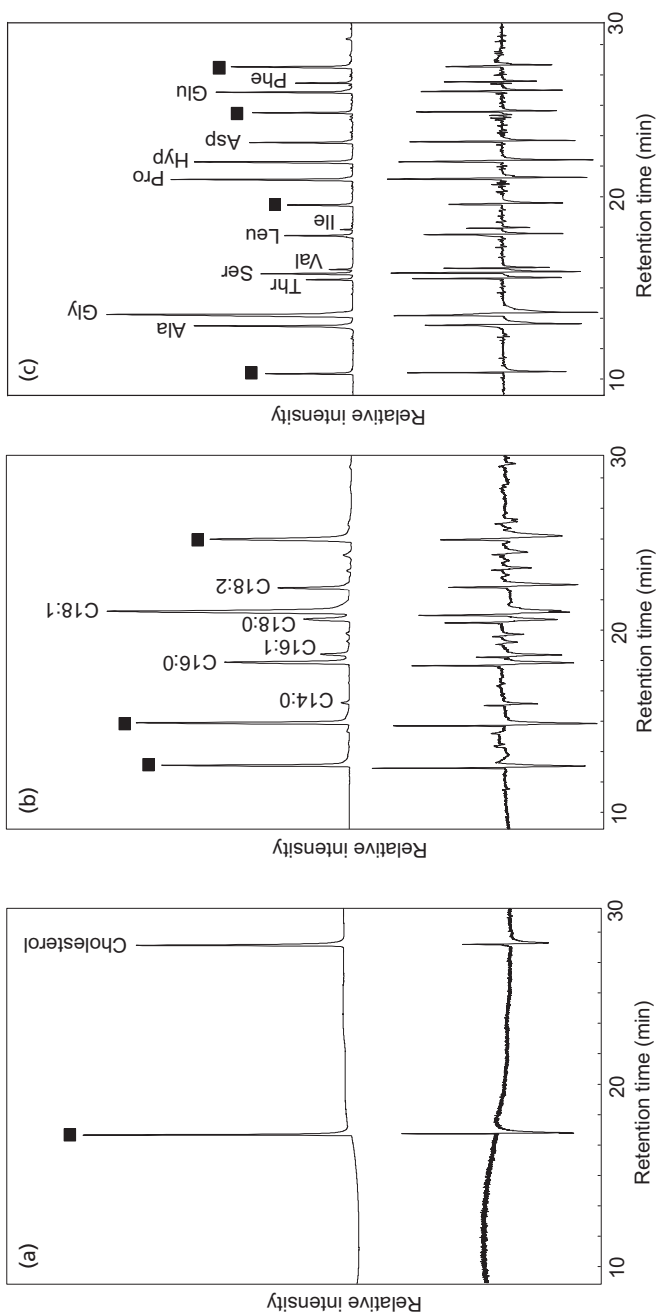


Figure 14.3 GC/C/IRMS chromatograms for biochemical components of pig bone from an isotopically controlled feeding experiment: (a) cholesterol (as its TMS ether), (b) fatty acids (as their methyl esters), and (c) amino acids (as their TFA-IPA derivatives). Peaks annotated with black squares are internal standards. (From Howland et al. 2003.)

One factor of particular importance is the position of the combustion reactor within the heated zone of the furnace. Altering this position may have a significant effect on determined δ values. This is probably due to the partial decomposition and/or pyrolysis of compounds eluting into the alumina reactor prior to the zone of optimal quantitative combustion. This problem may be reduced or resolved by moving the position of the reactor tube within the furnace. In addition, the fused silica capillary connecting the flow path from the GC to the reactor should be adjusted so that it extends beyond the metal connector to line the initial part of the reactor devoid of catalyst and insufficiently hot to enable quantitative combustion of eluting compounds. When making this modification ca. 5 mm of the polyimide coating the glass capillary should be removed, use of a gas burner is usually sufficient, to avoid deleterious effects caused by pyrolysis and/or combustion of the stationary phase.

One final important consideration is the degree by which compounds of interest have become enriched in the heavier isotope during the course of a labeling experiment. Analysis of highly isotopically enriched samples, as well as requiring a highly enriched reference, will require an adjustment to the amplification range of the middle Faraday cup corresponding to *m/z* 45 (or *m/z* 29) since high levels of ^{13}C or ^{15}N incorporation will quickly saturate the detector when set to a range suitable for natural abundance work. Commercially available GC/C/IRMS instruments now incorporate switchable resistors to enable rapid reconfiguration between amplification ranges. Since the linear range of the amplifiers of different instruments is variable, determining the point of saturation of the middle Faraday cup is of particular importance in determining accurate δ values of compounds from isotopically enriched labeling experiments.

Specific considerations for $\delta^{15}N$ analyses

In addition to the potential problem of isobaric interference from CO^+ ions (*m/z* 28) there are a number of additional factors that should be considered when attempting compound-specific $\delta^{15}N$ analysis. First, the actual abundance of N in an organic compound is typically <10% (cf. C > 60%), in a gas sample 1 molecule of N_2 contains two N atoms (only 1 in CO_2), the natural abundance of ^{15}N is 0.732% (cf. 1.08% ^{13}C) and N_2 has an ionization efficiency of 70% relative to that of CO_2. All of these factors result in the average N containing organic compound requiring, for $\delta^{15}N$ analysis, a minor isotope abundance around 50 times that required for the corresponding $\delta^{13}C$ analysis (Brand et al. 1994). This problem may be partially overcome by using shorter GC columns (e.g. 30 m) with both a smaller internal diameter (e.g. 0.25 mm) and thicker stationary phase (e.g. 0.4 μm) thereby generating a higher signal to noise ratio and enabling a higher sample loading. An increase in the sensitivity of the MS (e.g. a higher accelerating voltage, optimized tuning) will

also help alleviate this problem. The second major drawback relating to compound-specific $\delta^{15}N$ analyses results from the fact that the atmosphere comprises >78% N_2. The risk of introducing atmospheric N_2 into the ion source either by inadequate sample handling or leaks in the flow path of the GC/C/IRMS and thereby compromising the accuracy of determined $\delta^{15}N$ values is particularly high. Those parts of the GC/C/IRMS exposed to repetitive changes in temperature, i.e. those parts associated with the GC oven, are particularly prone to leaking and should be regularly checked and made gas tight. There can be no doubt that, despite its undeniable value in a wide range of applications, $\delta^{15}N$ analysis of individual compounds still remains a nontrivial methodology, which probably accounts for the current paucity of communications reporting the use of the technique.

Determining the δD and $\delta^{18}O$ values of individual compounds

The main difference between compound-specific δD and $\delta^{18}O$ analysis and the analytical techniques outlined above lies in the method by which the analyte gas is generated. For both δD and $\delta^{18}O$ analyses organic compounds are converted online to produce $^{1}H^{1}H$, $^{2}H^{1}H$, $^{12}C^{16}O$, $^{13}C^{16}O$, and $^{12}C^{18}O$. Several methods have been adopted by different manufacturers and researchers to achieve this chemical conversion, including reduction using a chromium metal catalyst at 1050°C (e.g. Gehre et al. 1996) and pyrolysis in a graphitized alumina reactor at 1450°C (Begley & Scrimgeour 1997; Tobias & Brenna 1997); only the latter technique shall be considered here.

Figure 14.2b is a schematic of a typical GC/C/IRMS instrument configured for compound-specific δD analysis. The combustion reactor has been replaced with a thermal conversion reactor (the term "thermal conversion" is used since there is currently no consensus on how to refer to this reaction and terms such as quantitative pyrolysis or calcination are too specific), which comprises an empty alumina tube maintained at a working temperature of 1450°C. At this temperature compounds eluting from the GC into the reactor are quantitatively converted to H_2, CO, and their corresponding isotopomers (after Burgoyne & Hayes 1998). Since the reaction produces no water the Nafion™ water trap can be omitted. The analyte gases are introduced to the mass spectrometer via an open split and the ion beams corresponding to *m/z* 2 and 3 are monitored by two Faraday cups for a simultaneous determination of H_2 and HD, respectively. An electrostatic filter is placed inline with the Faraday cup measuring the HD ion beam to remove the effect of the disperse $^{4}He^{+}$ ion beam that would otherwise superimpose and corrupt the measurement of the small HD beam (Hilkert et al. 1999). The resulting ion currents are recorded and converted to δD values in a process similar to that detailed above.

One crucial difference is the need to determine the effect that protonation reactions occurring in the ion source have on the determination of δD values.

Such reactions result in the formation of H_3^+ ions. Since the abundance of H_3^+ is proportional to the square of the hydrogen pressure a correction factor, termed an "H_3-Factor", may be determined based on the peak area information obtained from a series of reference gas pulses of different magnitude. The calculations of H_3-Factors and associated corrections have been reviewed previously in detail (Sessions et al. 2001a, 2001b).

Another vital factor for the determination of accurate δD values is the need to ensure that the alumina reactor in the thermal conversion interface has been suitably graphitized prior to performing any analyses. Burgoyne & Hayes (1998) reported that such treatment resulted in better peak shapes and reproducibility. This "conditioning" may be achieved by the direct injection of *n*-hexane or alternatively Bilke & Mosandl (2002) reported that conditioning the reactor by backflushing CH_4 resulted in greater stability over the ensuing sampling period. In addition, the accuracy of δD values may also be improved by the use of a concentrated "sacrificial" compound that elutes prior to the compound(s) of interest; this is a particularly useful method by which the accuracy of δD values determined for early eluting components may be improved. Finally, the flow through the reactor should ideally be fairly low, e.g. ca. 0.8 mL min^{-1} and certainly no greater than 1.2 mL min^{-1}, since an overly short residence time in the central ca. 10 cm zone of optimum performance can result in nonquantitative thermal conversion.

Determinations of compound-specific $\delta^{18}O$ values utilize an instrument configuration very similar to that used for δD analyses. Figure 14.2c depicts a schematic of a typical instrument configuration. The main point of divergence between the two techniques is in the design of the thermal conversion reactor. The empty alumina reactor is replaced by an alumina reactor containing a Pt tube that also contains Ni wire. The function of the Pt tube is to prevent any exchange of oxygen between the CO formed during thermal conversion and the alumina (Al_2O_3) reactor, the Ni is required as a support for graphitized carbon since carbon is not retained by the surface of the Pt tube. A sheath gas of He + 1% H_2 is applied as a counter flow between the alumina reactor and the platinum tube to prevent effluent flow returning towards the beginning of the reactor. Addition of 1% H_2 helps maintain reductive conditions in the reactor and improve overall stability. Eluting CO is introduced into a mass spectrometer equipped with a triple collector comprising three Faraday cups monitoring simultaneously *m/z* 28, 29, and 30 corresponding to the ions of the three isotopomers $^{12}C^{16}O$, $^{13}C^{16}O$, and $^{12}C^{18}O$, respectively (Hener et al. 1998; ThermoElectron 2003). $\delta^{18}O$ values are calculated in an analogous fashion to the methods outlined above. Of the four isotope techniques detailed above compound-specific $\delta^{18}O$ analysis is by far the most nascent and is likely to undergo many further developmental changes as the methodology is slowly adopted by the wider user community.

For an in depth review of this area see Meier-Augenstein (1999). In addition, readers are directed towards the ISOGEOCHEM list server

(http://list.uvm.edu/archives/isogeochem.html) as an invaluable resource for troubleshooting and the reporting of recent developments in GC/C/IRMS.

Data treatment: kinetic isotope effects (KIEs) and error analysis

Derivatization: general considerations

As discussed above, account must be taken of exogenous atoms added to the analyte molecule during analysis. In addition, any potential derivatization reaction must be evaluated for fractionation resulting from KIEs. Bonds involving heavier isotopes have a higher potential energy which can result in different reaction rates in reactions involving different isotopic species (Melander & Saunders 1980). For derivatization the most significant isotopic fractionation is the primary KIE that occurs at a specific molecular position causing an alteration in the δ value at that position. Hence, if the rate-determining step of a derivatization reaction involves the making or breaking of a bond to the atom under consideration, then the reaction involving that atom may be nonquantitative and any KIE must be quantified. If the KIE is reproducible, a correction can be made.

Correcting for derivative groups

If no KIE is present, the contribution of the derivative atom to the measured δ value of the derivatized compound can be calculated using a simple mass balance equation (14.1), where n is number of moles of the isotope of interest, F is the fractional abundance of the isotope of interest, c refers to compound of interest, d refers to the derivative group, and cd refers to the derivatized compound (Rieley 1994). For compounds at natural abundance, F can be replaced with the corresponding δ value (equation 14.2):

$$n_{cd}F_{cd} = n_cF_c + n_dF_d \quad (14.1)$$

$$n_{cd}\delta_{cd} = n_c\delta_c + n_d\delta_d \quad (14.2)$$

The application of these equations requires the isotope value of the derivatizing molecule to be established. If all the atoms of interest in the derivatizing reagent are transferred to the analyte, the contribution can be measured directly offline. However, if this is not the case or if the reagent is purchased in numerous small batches thereby precluding off-line analysis, the contribution of the derivatizing group can be measured indirectly by derivatizing a compound of known isotope value. However, this will have implications for the errors associated with the measurement, as discussed below.

Estimating KIEs

If the observed δ values for derivatized analytes do not equal those predicted by equation 14.1, then a kinetic isotope effect is present. Correction for the KIE can be made through the use of correction factors, as long as the KIE is proved to be reproducible across a range of analyte concentrations. These correction factors can be defined as the "effective" stable isotope composition of the derivative carbon introduced during derivatization taking into account the isotopic fractionation associated with the reaction. Correction factors are determined indirectly by measuring the δ value of an underivatized standard of the molecule of interest (by IRMS), the value of the standard after derivatization (by GC/C/IRMS), and using a rearranged equation 14.2 to determine δ_d. The δ_d term can then be replaced with δ_{corr} to represent the correction factor for the analyte of interest (Silfer et al. 1991; Macko et al. 1998).

Analysis of errors

Where no KIE is present, the measurement of the δ value is a result of two measurements, each with their own associated precision. During correction for the added derivative carbon, where σ is the standard deviation associated with a given δ determination, the errors propagate according to equation 14.3.

$$\sigma_c^2 = \sigma_{cd}^2\left(\frac{n_c + n_d}{n_c}\right)^2 + \sigma_d^2\left(\frac{n_d}{n_c}\right)^2 \tag{14.3}$$

In derivatization reactions with a KIE, correction factors are first calculated, this calculation introduces another step where errors propagate. The propagation of errors under these circumstances is calculated using equation 14.4, where subscript s stands for the standard used in correction factor determination and sd stands for the derivatized standard. The magnitude of the errors associated with the correction factors themselves can be calculated using equation 14.3, along with the precisions for each determination (Docherty et al. 2001).

$$\sigma_c^2 = \sigma_d^2\left(\frac{n_s}{n_c}\right)^2 + \sigma_{ad}^2\left(\frac{n_a + n_d}{n_c}\right)^2 + \sigma_{cd}^2\left(\frac{n_c + n_d}{n_c}\right)^2 \tag{14.4}$$

The precision associated with the determination of δ values is derived from the errors associated with the correction factors and is also dependent on the molar ratio of the element of interest between the sample and derivative. Therefore in order to minimize the final error it is preferable to employ a derivatization reaction with no associated KIE and to minimize the amount of derivative atoms added.

Specific considerations when working at high stable isotopic enrichments

In recent years, the use of highly enriched stable isotope tracers in "pulse-chase" type experiments has become far more widespread since they circumvent the considerable loss of sensitivity that can be experienced through depletion of natural abundance tracer levels as a result of respiration, low incorporation rates, and dilution effects. Whilst highly enriched tracers have many benefits, there are also potential pitfalls associated with interpretation of the resulting data. Carryover within a run has been demonstrated to have a significant effect on the $\delta^{13}C$ values reported for compounds eluting immediately after a highly enriched component (Mottram & Evershed 2003). Whilst this effect does not represent a serious drawback for qualitative studies, careful consideration is needed when quantitatively interpreting results from analysis of complex mixtures containing enriched compounds. The recommendation here is that experiments resulting in excessively enriched compounds are probably of questionable value except for rather specific investigations where dilution effects are likely to be very substantial.

Applications of compound-specific stable isotope approaches in ecology and paleoecology

The application of GC/C/IRMS in organic geochemical studies

The major impetus for the development of the GC/C/IRMS technique came from the work of Hayes and co-workers during the 1980s and 1990s, such that the vast majority of early instruments were acquired by organic geochemistry laboratories. The range of applications of GC/C/IRMS in this area has been truly spectacular, and such a volume of published works makes a comprehensive review beyond the scope of a contribution of this nature. Thus, what follows is a brief overview of the applications of GC/C/IRMS in this area, with specific papers being used to highlight areas of novel application.

A variety of processes, including environmental conditions during organic matter production (Bidigare et al. 1997; O'Leary 1981), diagenesis (e.g. Spiker et al. 1985), catagenesis (e.g. Hayes et al. 1992), and heterotrophic reworking (Hayes et al. 1990; Ostrom & Fry 1993; Logan et al. 1997) all exert significant controls on the carbon isotopic composition of sedimentary organic matter. Although this presents the opportunity for the application of stable carbon isotope methodologies to diverse investigations, it also makes it difficult to interpret the bulk organic carbon isotope record; consequently, the use of GC/C/IRMS to determine the carbon isotopic compositions of specific compounds has become widely used in organic geochemistry. Using this approach, primary vs. secondary and allochthonous vs. autochthonous organic materials can be distinguished based on their isotopic compositions.

Moreover, the information recovered from a single sample is much more diverse: insights into algal photosynthesis, higher plant community structure, and bacterial recycling of organic matter can be elucidated from a few analyses (Freeman et al. 1990). However, the compound-specific approach also introduces additional complications related to carbon isotope fractionation during the biosynthesis of specific compound classes (e.g. Lockheart et al. 1997; Schouten et al. 1998).

The utility of compound-specific isotope analysis is directly related to the specificity of the compounds being analyzed. Compounds with very specific sources, such as alkenones derived from certain species of haptophyte algae, provide more precise data, allowing the most constrained interpretation (e.g. Pagani et al. 1999), but less diagnostic compounds can still provide useful information in the proper context. The lipids of a range of organisms (including marine and lacustrine algae, bacteria, archaea, and higher plants), their degradation pathways and representative biomarkers in the geologic record have been studied for decades, and this literature is only briefly summarized here, with particular emphasis on compounds used in carbon isotope studies.

Alkenones, long-chained (C_{37}–C_{39}) unsaturated ethyl and methyl ketones produced by only a few species of Haptophyte algae in the modern ocean (Volkman et al. 1980; Marlowe et al. 1984) are the most commonly used algal biomarkers in environmental investigations due to their relative ease of preparation and isotopic analysis, source specificity, diagenetic robustness, and the use of their distributions as a sea-surface temperature proxy. One of the most striking applications of alkenone $\delta^{13}C$ values has been to the development of atmospheric pCO_2 records from the Eocene to the Miocene (Pagani et al. 1999, 2005). Pagani and colleagues investigated sediments from ocean gyres, where growth rate variations were thought to be minor. Thus, changes in alkenone (and carbonate) derived ε_p values could be interpreted as changes in pCO_2.

After alkenones, steroids are the algal lipids most commonly investigated using isotopic approaches. Because of their structural diversity (Volkman 1986; Volkman et al. 1998), certain sterols are relatively diagnostic for specific compound classes. For example, 24-methylcholesta-5,22*E*-dien-3β-ol and especially 24-methylcholesta-5,24(28)-dien-3β-ol have both been invoked as diatom biomarkers, although these sterols are also present in other algae (Volkman et al. 1998). More diagnostic are the 4-methylsterols, especially 4α,23,24-trimethyl-5α-cholest-22*E*-en-3β-ol (dinosterol), as biomarkers for dinoflagellates (Withers et al. 1979). Several workers have determined sterol $\delta^{13}C$ values in modern surface-waters and shallow marine sediments (Pancost et al. 1997, 1999; Popp et al. 1989; Eek et al. 1999). Sterol $\delta^{13}C$ values have been used to either clarify the sterol sources (e.g. that 24-ethylcholesterols in Peru surface-waters derive from diatoms and not higher plants; Pancost et al. 1999), or evaluate controls on algal growth rates (Pancost et al. 1997).

In ancient sediments, steranes are among the most abundant preserved hydrocarbons; however, thermal isomerization typically results in a complex distribution of steranes and determination of specific compounds' $\delta^{13}C$ values is difficult (e.g. Schouten et al. 2000).

Chlorophylls and their degradation products can also be used as tracers for the isotopic composition of primary photosynthate. Since they are not GC-amenable, it is difficult to directly determine chlorophyll (and porphyrin) $\delta^{13}C$ values, and most efforts have focused on their degradation products. Phytol, the esterified side chain of most chlorophylls, and its inferred hydrocarbon degradation products (pristane, phytane) have been the subject of compound-specific carbon isotopic analysis since the advent of the technique (Freeman et al. 1990; Hayes et al.1990). Maleimides, 1-*H*-pyrrole-2,5-diones, are direct degradation products of the chlorophyll and bacteriochlorophyll tetrapyrrole structure, and are common in extracts of ancient sediments and are GC-amenable (Grice et al. 1997). Thus, maleimide $\delta^{13}C$ values are relatively easy to determine and have been used to gain insight into carbon cycling in ancient settings (e.g. Permian Kupferschiefer; Grice et al. 1997).

Long-chain *n*-alkyl compounds are major components of epicuticular waxes from the leaves of vascular plants (Eglinton et al. 1962). These compounds, especially *n*-alkanes, are relatively resistant to degradation, which makes them suitable for use as higher plant biomarkers (Cranwell 1981). It is relatively easy to determine carbon isotopic compositions of higher plant *n*-alkyl compounds using GC/C/IRMS, because they commonly occur in high abundances and relatively simple adduction procedures can be used to obtain pure fractions; thus, the carbon isotopic compositions of these compounds in modern plants, soils, and lacustrine and marine sediments have been extensively studied. Another higher plant derived component that is commonly investigated is lignin, a relatively stable and microbially resistant heteropolymeric structure comprising phenyl-propanoid subunits (Sarkanen & Ludwig 1971) and a significant component of wood. Moreover, lignin monomers have different sources (Hedges & Parker 1976) such that the isotopic compositions of syringyl, vanillyl and cinnamyl phenols can be used to distinguish isotopic signals of different plant types (e.g. Huang et al. 1999). Although preparation of lignin monomers for isotopic analysis requires careful chemical work-up, typically involving CuO oxidation of the lignin macromolecule, generated fractions are readily analyzable by GC-C-IRMS (Goñi & Eglinton 1996).

Sedimentary higher plant biomarker $\delta^{13}C$ values are commonly used to identify shifts in the relative proportion of C3 vs. C4 plants in the adjacent terrestrial ecosystem (e.g. Bird et al. 1995; Yamada Ishiwatari 1999; Huang et al, 2000). Often subtle changes can be identified, but also dramatic isotopic shifts in *n*-alkane $\delta^{13}C$ values have been observed and used to infer significant changes in past ecosystems. Freeman & Colarusso (2001) revisited Bengal Fan sediments that had previously been investigated using bulk sedimentary $\delta^{13}C$

values (France-Lanord & Derry 1994); observing a shift in *n*-alkane $\delta^{13}C$ values from −30 to −22‰, they confirmed the shift from C3-dominated to C4-dominated vegetation on the Himalayan foreland. The same approach was utilized in Mesozoic sediments from the southern proto North Atlantic (Kuypers et al. 1999), but was feasible only because sulfurization of algal organic matter had prevented dilution of terrestrial *n*-alkanes with marine-derived counterparts. In DSDP Site 367, *n*-alkane $\delta^{13}C$ values exhibited a dramatic shift of ca. 14‰ at the Cenomanian=Turonian boundary (ca. 90 Ma), suggesting a nearly complete vegetation shift in parts of North Africa at this time (Kuypers et al. 1999). This was attributed to a pCO_2 decrease associated with the C=T oceanic anoxic event but was nonetheless surprising as the C4 photosynthetic pathway was not thought to have evolved by this time.

The most common bacterial biomarkers in marine sediments are free and bound (phospholipid) fatty acids, of which the latter comprise the membranes of bacteria; however, eukaryotes also contain membranes comprised of phospholipid fatty acids and these compounds are not diagnostic as a class. The most common, such as saturated C_{16} and C_{18} fatty acids, are particularly widespread and appear to have little utility as tracers of explicit prokaryotic processes. However, some fatty acids, characterized by site-specific methyl groups, double bonds, or cyclic moieties are less common. Other bacterial membrane lipids are the hopanoids, pentacyclic triterpenoids common in a range of primarily aerobic bacteria (Ourisson et al. 1987; Farrimond et al. 2000). Many hopanoids are well resolved during GC analysis of marine sediments and their $\delta^{13}C$ values can be readily determined.

In addition to the above widespread bacterial biomarkers, a variety of compounds are biomarkers for photosynthetic bacteria. These include diverse pigments, including isorenieratene (Liaaen-Jensen 1978), chlorobactene, and bacteriochlorophylls *d* and *e*, which are diagnostic for green sulphur bacteria and thus, photic zone euxinia in the depositional environment. Of these, it is relatively easy to determine $\delta^{13}C$ values for isorenieratene derivatives (especially isorenieratane) due to their high molecular weight and long retention time eliminating most co-elution problems. Because green sulphur bacteria utilize the reverse tricarboxylic acid (TCA) cycle during carbon assimilation, their $\delta^{13}C$ values are less depleted relative to other organic matter (Quandt et al. 1977; Sirevag et al. 1977). Combined with the structural specificity of isorenieratene, this provides confirmation of the presence of green sulphur bacteria and, critically, photic zone euxinia, in paleoceanographic investigations spanning Earth history (e.g. Koopmans et al. 1996).

Compound-specific isotope analysis of bacterial and archaeal lipids has been particularly useful when applied to the investigation of anaerobic oxidation of methane. Determination of extremely low $\delta^{13}C$ values (−80 to −120‰) for archaeal (archeol, hydroxyarcheol and GDGTs) and sulphate reducing bacterial membrane lipids (including fatty acids) in cold seep sediments has been used to confirm the role of these organisms in the anaerobic oxidation

of methane at cold seeps (Hinrichs et al. 1999; Thiel et al. 1999; Pancost et al. 2001; Elvert et al. 2001). The low $\delta^{13}C$ values offer direct evidence that the Archaea and sulfate-reducing bacteria (SRB) are incorporating methane. A wide range of settings and compounds have now been investigated, with almost all revealing a predominance of archaeal lipids (e.g. Figure 14.4), confirming the important role of these organisms in methane cycling.

The application of GC/C/IRMS in archeology

Organic residues in archeological pottery

Early work in the use of stable isotopes in archeological pottery involved bulk isotopic analysis (e.g. Hastdorf & DeNiro 1985; Morton & Schwarcz 1988; Sherriff et al. 1995). Compound-specific stable isotope analysis was first applied to archeological potsherds in 1994 (Evershed et al. 1994), confirming that an extract with lipid distribution consistent with wild type *Brassica* species was of a C3 plant origin. The application of compound-specific stable isotope analysis via GC/C/IRMS to lipid residues in archeological pottery allows greater specificity to be achieved than is possible with bulk analyses, since the structures of diagnostic (biomarker) components of complex mixtures can be unambiguously linked to their stable isotope values. Thus, compound-specific stable isotope analysis avoids ambiguities arising from contamination (e.g. plasticizers originating from plastic bags in which sherds are often stored), which cannot be resolved from endogenous components in bulk isotope analyses. Compound-specific $\delta^{13}C$ values also afford insights into the biochemical sources of carbon even when chemical structures are identical. $\delta^{13}C$ values of fatty acids provide the basis for distinguishing between ruminant (e.g. sheep/goat and cattle) and porcine (pig) adipose fats. The potential of GC/C/IRMS in this area (Table 14.2) was realized by Evershed and co-workers in 1997; $\delta^{13}C$ values of the $C_{16:0}$ and $C_{18:0}$ fatty acids in medieval lamps and dripping dishes, when compared with modern reference fats, revealed that ruminant fat had been used as fuel in the lamps, whereas porcine fat was collected in the dripping dishes, disproving the theory that fat was collected from spit-roasting and recycled as lamp fuel (Evershed et al. 1997; Mottram et al. 1999). The $\delta^{13}C$ values exhibited by these animals reflect their different diets and variations in their metabolisms and physiologies (Evershed et al. 1999)

Ruminant adipose and dairy fats can also be distinguished by the $\delta^{13}C$ values of their fatty acids (Dudd & Evershed 1998). The $C_{18:0}$ fatty acid in dairy fat is significantly more depleted in ^{13}C (ca. 2.1‰; Copley et al. 2003). Fatty acids in ruminant adipose are mainly synthesized from acetate (as acetyl CoA), originating predominately from the fermentation of dietary carbohydrate in the rumen. The mammary gland is incapable of biosynthesizing the $C_{18:0}$ fatty acid; instead, it is obtained via the remobilization of adipose

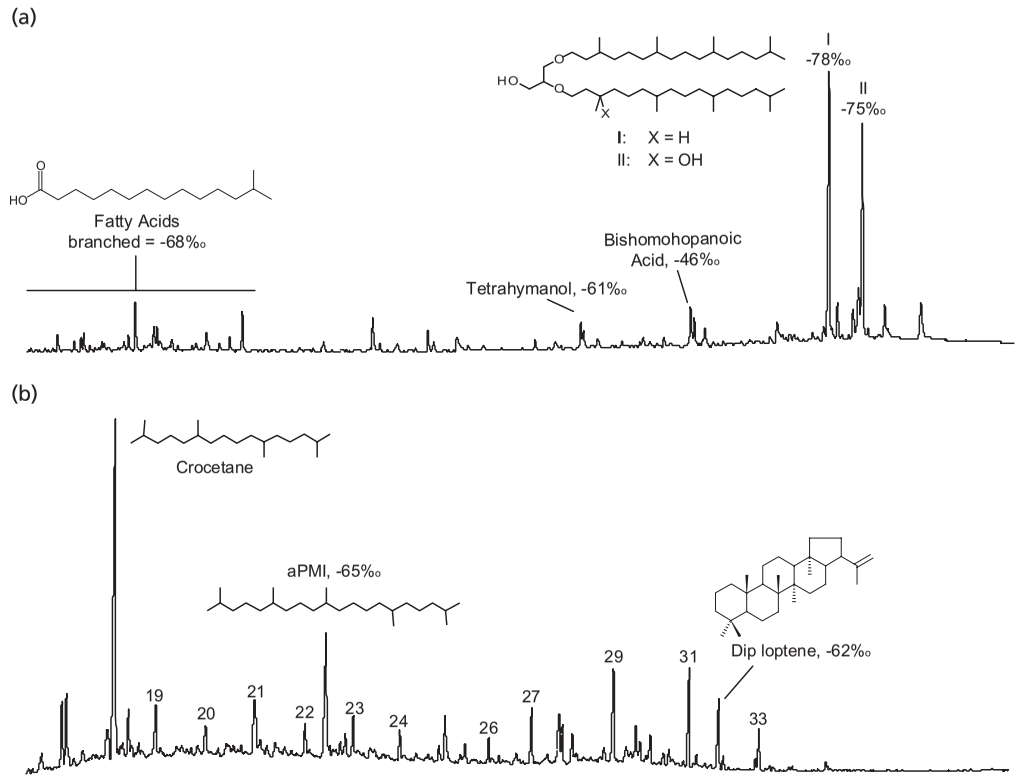


Figure 14.4 Partial gas chromatograms showing the relative abundances of archaeal and bacterial biomarkers (and their carbon isotopic compositions) in the polar fraction (a) and apolar fraction (b) of the total lipid extract of a Napoli Mediterranean mud volcano cold seep. (Adapted from Pancost et al. 2000; Pancost & Sinninghe Damsté 2003.)

Table 14.2 Summary of compound-specific stable isotope analyses applied to archeology.

Object or find type	Biomarker	Uses	References
Potsherd	$C_{16:0}$ and $C_{18:0}$ fatty acids	Distinguishes between remnant animal fats of ruminant adipose, ruminant dairy or porcine adipose origin	Evershed et al. 1997, 2003; Mottram et al. 1999; Dudd & Evershed 1998; Copley et al. 2003, 2005a,b,c; Berstan et al. 2004; Craig et al. 2005
	n-Dotriacontanol	Biomarker for maize processing	Reber et al. 2004 Reber & Evershed 2004a,b
Bone lipid	Cholesterol	Indicator of $\delta^{13}C$ value of whole diet. Depleted in ^{13}C relative to whole diet. Neosynthesis > assimilation of dietary cholesterol in governing $\delta^{13}C$ value of bone cholesterol. Indicator of short-term diet.	Stott & Evershed, 1996; Stott *et al.*, 1997b; Jim *et al.*, 2001, 2004
	Fatty acids	Good indicators of general short-term trends in diet.	Howland et al. 2003; Copley et al. 2004
	Nonessential fatty acids	$\delta^{13}C$ values correlates well with whole diet.	
	Linoleic acid	$\delta^{13}C$ value correlates well with dietary linoleic acid.	
Bone protein	Amino acids:		
	Alanine, glycine, threonine, serine, valine, leucine, isoleucine, proline, hydroxyproline, aspartate, glutamate, phenylalanine	Mass balance calculations using the $\delta^{13}C$ values of single amino acids accurately predicts $\delta^{13}C$ value of whole collagen	Howland et al. 2003
	$\Delta^{13}C_{Glycine\text{-}Phenylalanine}$	Marine dietary indicator	Corr et al. 2005
	Alanine and glutamate	$\delta^{13}C$ values from bone collagen correlate well with whole diet	Howland et al. 2003
	Leucine and phenylalanine	Little isotopic fractionation between diet and bone collagen	Howland et al. 2003
	Leucine	Long-term indicator of the protein component of diet	Copley et al. 2004
	Glutamate	Long-term whole diet indicator providing an internal check of apatite	Copley et al. 2004

fatty acids and directly from the dietary C_{18} fatty acids, after biohydrogenation in the rumen (Moore & Christie 1981). The difference between the $C_{18:0}$ fatty acids from ruminant adipose and dairy fat can be explained by the fact that lipids are more depleted in ^{13}C than carbohydrates (DeNiro & Epstein 1977) and approximately 60% of the $C_{18:0}$ fatty acid in dairy fat is derived via biohydrogenation of dietary unsaturated C_{18} fatty acids (i.e. $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) in the rumen. The $\delta^{13}C$ values of the contributors to the $C_{18:0}$ fatty acid in dairy fat are summarized in Figure 14.5. Compound-specific $\delta^{13}C$ values are readily determined by GC/C/IRMS for fatty acids derivatized to FAMEs.

GC/C/IRMS analysis of remnant animal fats of archeological origin has now been extensively used to address some key questions concerning animal husbandry in prehistory, for example the earliest evidence for dairying in prehistoric Britain (Dudd & Evershed 1998; Copley et al. 2003, 2005a, 2005b, 2005c), and the exploitation of pigs in the late Neolithic (Mukherjee, 2004; Mukherjee et al. in press). $\delta^{13}C$ values of fatty acids extracted from potsherds have also been used to identify prehistoric dairying activities in the Western Isles of Scotland (Craig et al. 2005).

Clearly many archeological vessels will have been used to process commodities from more than one type of animal. In order to account for this a mixing model is used to calculate theoretical $\delta^{13}C$ values. This mathematical model has been used elsewhere for the detection of the mixing of vegetable oils of differing stable carbon isotope composition (Woodbury et al. 1995; Mottram et al. 2003) and sedimentary lipids (Bull et al. 1999) and utilises the percentage abundance of each specific fatty acid and its associated $\delta^{13}C$ value:

$$\delta^{13}C_{mix} = \delta^{13}C_{(A)}\left(\frac{(X \times A)}{(X \times A)+(Y \times B)}\right) + \delta^{13}C_{(B)}\left(\frac{(Y \times B)}{(X \times A)(Y \times B)}\right) \quad (14.5)$$

where $\delta^{13}C_{mix}$ is the predicted $\delta^{13}C$ value of the fatty acid with contributions from fats *A* and *B*, $\delta^{13}C_{(A)}$ is the $\delta^{13}C$ value of the individual fatty acid in fat *A*, $\delta^{13}C_{(B)}$ is the $\delta^{13}C$ value of the individual fatty acid in fat *B*, *X* is the percentage of fat *A* present, *Y* is the percentage of fat *B* present, *A* is the percentage of the individual fatty acid in fat *A*, and *B* is the percentage of the individual fatty acid in fat *B*.

Compound-specific stable carbon isotope analysis of bone biochemical components

The use of bone in paleodietary reconstruction was motivated by the variations in collagen $\delta^{13}C$ values observed during ^{14}C dating, and light stable isotopes were first used in archeology in the late 1970s (Vogel & Van der Merwe 1977). Since then dietary analysis through both carbon and nitrogen isotopes

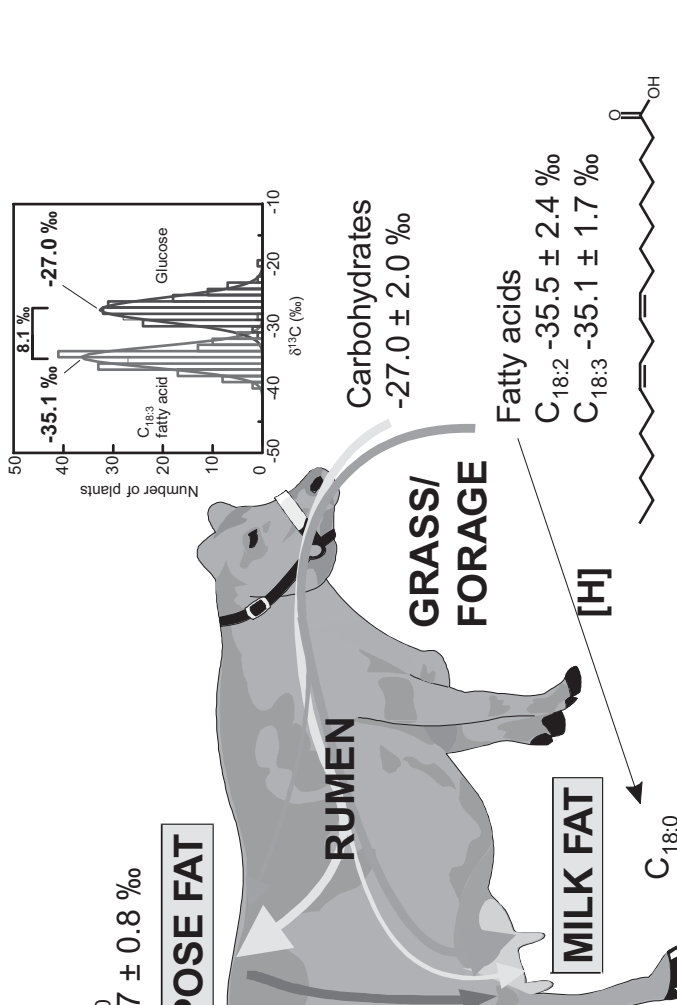


Figure 14.5 Diagram showing the routing of dietary fatty acids and carbohydrates in the rumen, adipose tissue, and mammary gland of the ruminant animal and histogram of the $\delta^{13}C$ values of $C_{18:3}$ fatty acid and glucose extracted from plants demonstrating the 8.1‰ mean difference between them. (Adapted from Copley et al. 2003; Docherty 2002.)

has been applied extensively to archeological studies. The reconstruction of ancient diets is possible because $\delta^{13}C$ and $\delta^{15}N$ values of fossil bone reflect the isotopic signatures of the local environment, specifically the plants that lie at the base of the food chain (Gannes et al. 1998).

Very extensive investigations of human diet in the past have been undertaken using the stable isotopic signals recorded in collagen and hydroxyapatite in bones, with important evidence also coming from teeth. Although isotopic analysis may not always enable the precise reconstruction of an animal's diet, it does allow discrimination of animals belonging to particular dietary niches (Gannes et al. 1998). Compound-specific stable carbon isotope analysis of bone components such as cholesterol, fatty acids, and individual collagen amino acids is finding increasing application to paleodietary reconstruction. An application of compound-specific isotopic analyses is in improving our understanding of the relationship between dietary macronutrient composition and the $\delta^{13}C$ values of bone components (Hare et al. 1991; Ambrose 1993; Jim et al. 2001, 2003b, 2004, 2006).

Cholesterol in archeological bone

The use of cholesterol as a paleodietary indicator is a relatively recent development. Cholesterol was demonstrated to survive in archeological bone in 1995 (Evershed et al. 1995). Archeological human and animal bones, including a 75,000 year old whale bone from a permafrost deposit, were found to contain free cholesterol and cholesteryl fatty acyl esters, and diagenetic products (5α- and 5β-cholestan-3-one, 5α- and 5β-cholestanol and cholest-5-en-7-one-3β-ol; Evershed et al. 1995; Stott et al. 1997a). The cholesterol found in bone may derive from either the remnants of the original bloodborne lipid (in the case of vascular bones), the fat component of bone marrow that would have been present at the time of death of the organism, or a component of cellular lipids present in bone-forming cells (Stott et al. 1997a). The use of cholesterol as a paleodietary indicator has been extensively investigated (Stott & Evershed 1996; Stott et al. 1997b, 1999; Jim et al. 2004). The $\delta^{13}C$ values of cholesterol are readily recorded by GC/C/IRMS as its TMS ether derivative (Figure 14.3) and were constant across different skeletal members for a given individual (Stott & Evershed 1996). Assessment of $\delta^{13}C$ values for cholesterol from animals raised on isotopically distinct diets (Stott et al. 1997b; Jim, 2000; Jim et al. 2001, 2003b, 2004; Corr 2003), indicate that: (i) cholesterol is a good indicator of whole diet, (ii) neosynthesis of cholesterol is more significant than assimilation in determining the $\delta^{13}C$ value of cholesterol, and (iii) bone cholesterol $\delta^{13}C$ values respond to changes in the isotopic composition of whole diet more rapidly than collagen and apatite such that cholesterol is an indicator of short-term diet (Stott et al. 1997a; Jim, 2000). These results have been applied, alongside collagen and apatite analysis, to address archeological questions relating to the diets of a range of

ancient populations (Stott et al. 1999; Jim, 2000; Jones, 2002: Corr 2003; Howland, 2003; Copley et al. 2004).

Fatty acids in archeological bone and those of experimental animals

As discussed above $C_{16:0}$ and $C_{18:0}$ fatty acids have been used as biomarkers for animal fats in the study of lipid residues in archeological pottery sherds. However, the analysis of fatty acids extracted from archeological bone has been much less explored (Evershed et al. 1995). This is mainly due to the low survival of bone fatty acids in the archeological record; fatty acids only seem to be preserved in significant abundances under exceptional burial environments, for example arid and waterlogged sites (Evershed & Connolly 1987; Copley et al. 2004). Fatty acids present in bone mainly derive from bone marrow fat (Evershed et al. 1995). Studies on rats and pigs raised on isotopically controlled diets have shown that bone fatty acid $\delta^{13}C$ values are ^{13}C-depleted by up to 3.4‰ with respect to whole diet (Jim, 2000; Jim et al. 2001, 2003b; Howland et al. 2003). This phenomenon results from a kinetic isotope effect occurring during the oxidation of pyruvate by pyruvate dehydrogenase to acetyl CoA, the common precursor in lipid biosynthesis (DeNiro & Epstein 1977; Hayes 1993). The $\delta^{13}C$ values of bone fatty acids have recently been used together with those of individual amino acids and apatite as indicators of trends in the management of domesticated animals in Egypt (Copley et al. 2004).

Amino acids in archeological bones and those of experimental animals

Compound-specific stable isotope analyses of the building blocks of complex biopolymers, such as collagen, are essential to unraveling the stable isotope signals expressed in bulk protein signals. The exploitation of individual collagenous amino acids has great potential in paleodietary reconstruction, however, surprisingly only a handful of studies have determined the $\delta^{13}C$ values of amino acids from ancient bone collagen (Hare & Estep 1983; Tuross et al. 1988; Hare et al. 1991; Fogel & Tuross, 2003; Jim et al. 2003b; Copley et al. 2004; Corr et al. 2005). Amino acids are difficult to isolate for isotopic analysis. Ion exchange liquid chromatography has been used but is slow and the reproducibility of retention times is poor (Hirs et al. 1954, Gaebler et al. 1966, Hare & Estep 1983, Macko et al. 1983, 1987, Tuross et al. 1988, Hare et al. 1991). Moreover, van Klinken (1991) has reported that isotopic fractionation is a major disadvantage with this technique.

GC/C/IRMS enables amino acids to be separated by GC and combusted on-line, for stable isotopic analysis, thereby avoiding manual preparative steps (see Figure 14.3). The $\delta^{13}C$ values of individual collagen amino acids are highly robust and by use of mass balance calculations can even be used to reconstruct the bulk $\delta^{13}C$ value of whole collagen (Jim et al. 2003a). A

recent study of pigs raised on six isotopically controlled diets investigated the routing of dietary macronutrients to bone biochemical components (Howland et al. 2003), showing that: (i) the $\delta^{13}C$ values of single amino acids accurately predicted the $\delta^{13}C$ value of whole collagen; (ii) the $\delta^{13}C$ values of nonessential amino acids alanine and glutamate from bone collagen correlated well with whole diet; and (iii) the essential amino acids leucine and phenylalanine showed little isotopic fractionation between diet and bone collagen. Still more recently a feeding experiment involved rats fed on diets where the $\delta^{13}C$ values of the major dietary macronutrients were switched between C3 and C4 enabling quantitative assessment to be made of the carbon sources used in the *de novo* synthesis of nonessential amino acids (Jim et al. 2006).

Applying these techniques to archeological populations enabled Evershed and co-workers to demonstrate the wider utility of compound-specific stable isotope analysis of individual bone collagen amino acids to distinguish between high marine protein and terrestrial consumers (Corr et al. 2005). This was previously shown to be problematic in extremely arid environments using bulk collagen $\delta^{15}N$ values alone (Heaton 1987; Heaton et al. 1986; Schwarcz et al. 1999; Sealy 1997) since herbivore bone collagen $\delta^{15}N$ values overlap with the range for marine species (Heaton et al. 1986; Sealy et al. 1987). Due to their contrasting metabolic pathways, $\delta^{13}C$ values of the essential amino acid phenylalanine and the nonessential amino acid glycine in bone collagen preserve different paleodietary signals and this difference ($\Delta^{13}C_{Glycine\text{-}Phenyalanine}$) can be exploited to distinguish between high marine protein and terrestrial consumers (Figure 14.6). Compound-specific stable isotope analysis of single collagenous amino acids along with fatty acids, collagen, and apatite have been used to investigate foddering and foraging strategies of domesticated animals from Qasr Ibrim, Egypt (Copley et al. 2004). Essential and nonessential collagenous amino acids provided long-term indicators of the diet of cattle and sheep/goat. The essential amino acid leucine, incorporated directly from dietary protein (Beynon 1998), has a $\delta^{13}C$ value that directly reflects the $\delta^{13}C$ values of the leucine in the protein component of the plants that are incorporated into animals' diets.

The recent investigations discussed above now mean that a range of bone-based biochemical proxies exists for investigating: (i) whole diet, (ii) specific elements of the diet, e.g., protein and energy components, and (iii) long- and short-term dietary variation within such proxies, and as such are highly applicable to the investigation of ancient diet.

^{13}C-labeling of lipids to investigate environmental microbes

The complexity of the microbial populations in sedimentary environments is universally acknowledged, with major challenges to their study arising from the unculturable nature of the major proportion of such populations (Amann

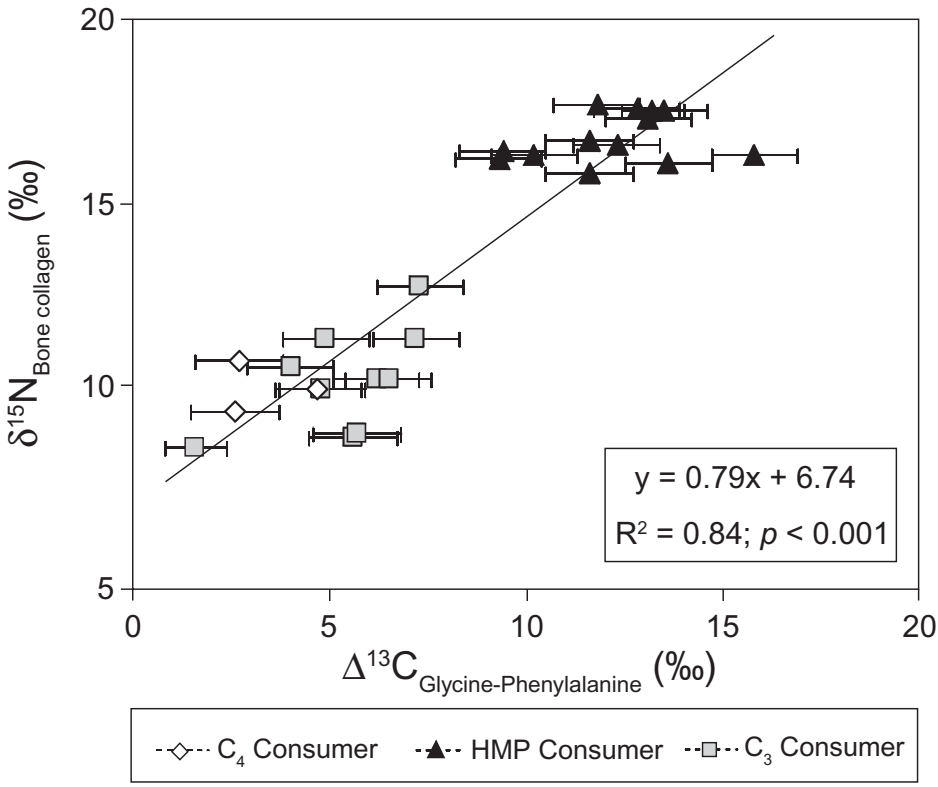


Figure 14.6 Plot showing the correspondence between $\Delta^{13}C_{gly\text{-}phe}$ and $\delta^{15}N$ values of collagen from hunter/gatherers from the South western Cape, indicating the potential of compound-specific glycine carbon isotope values as new marine dietary proxy. (Adapted from Carr et al. 2005.)

et al. 1995). The use of compound-specific stable isotope approaches to study living microbial populations rests on matching specific biomarkers, to a particular group(s) of organism(s). The biomarkers chosen are required to indicate both the presence and the activity of a given microbial group, therefore, on cell death, that biomarker must be rapidly removed from the system. For this reason the majority of studies focus on microbial membrane lipids, which have been shown to be rapidly degraded on cell death. Combining biomarker analysis with isotopic labeling studies provides the opportunity of linking specific processes, e.g. methane-oxidation, with the microbial taxa responsible. Labeling approaches involve adding a ^{13}C-labeled substrate to a microcosm, sediment, soil, or culture medium, then following its fate temporally

and/or spatially. Table 14.3 lists examples of the application of compound-specific stable isotopes in this area.

A major area of utility of this methodology is the use of $^{13}CH_4$ to investigate methanotrophic bacteria in both aerobic and anaerobic environments, including sediments (Boschker et al. 1998; Nold et al. 1999), soils (Bull et al. 2000; Crossman et al. 2001, 2004, 2006; Knief et al. 2003), microbial mat (Blumenberg et al. 2005), and peat bogs (Raghoebarsing et al. 2005). A major advantage of using methane arises from its ease of addition to microcosms providing the opportunity to target such an important group of microorganisms. This approach was especially effective in investigating unculturable high-affinity methanotrophs in soils revealing novel type II methanotrophs producing a br17:0 PLFA (Bull et al. 2000). In addition to the classification of bacteria, Crossman et al. (2004) investigated how communities adapted to their environment. They used $^{13}CH_4$ incubations in laboratory microcosms to demonstrate variations in methanotrophic bacterial populations with depth through a landfill cover soil (Figure 14.7); type I methanotrophs were found to be more active in the surface layers, where concentrations of oxygen were highest and methane concentration low, while type II methanotrophs dominated in the deepest layers of the cap where methane concentrations were high and oxygen low.

Other research has also exploited the fact that the $\delta^{13}C$ value of biogenic methane is highly depleted (−50 to −100‰) as a result of isotopic fractionation (Whiticar et al. 1986) and there have been a variety of reports of the presence of ^{13}C-depleted archaeal ether lipids and sulphate-reducing bacterial biomarkers in marine sediments near methane seeps (Pancost et al. 2001; Teske et al. 2002), mud volcanoes (Pancost et al. 2000), carbonates (Thiel et al. 1999, 2001) and in the water column (Schouten et al. 2001). These have been interpreted as communities of methane-consuming archaea, possibly methanogenic archaea operating in reverse, and sulphate-reducing bacteria performing anaerobic methane oxidation and are reviewed in greater detail by Pancost & Sinnighe-Damsté (2003).

Microbial communities utilizing products of organic matter mineralization have also been investigated (Boschker et al. 2001; Pombo et al. 2002, 2005). For example, Boschker et al. (2001) have shown through laboratory incubation of small anoxic/brackish sediment cores that ^{13}C-acetate and ^{13}C-propionate were utilized by different members of the microbial community. ^{13}C-label from acetate was recovered mainly from even carbon numbered PLFAs (16:1ω7c, 16:0, 18:1ω7c) while primarily odd carbon numbered fatty acids (*a*15:0, 15:0, 17:1ω6, 17:0) became labeled upon incubation with propionate. These findings clearly indicate that the two substrates were predominantly consumed by different specialized groups of sulfate-reducing bacteria. The PLFA labeling pattern for the acetate consumers was similar to *Desulfotomaculum acetoxidans* and *Desulfofrigus* spp., two acetate-consuming sulfate-reducing

Table 14.3 Summary of compound-specific stable isotope based investigations of environmental microbes.

Environment	^{13}C-labeled substrate	Biomarker	Detection method	Conclusion	Reference
Laboratory microbial cultures	Natural abundance glycerol, glucose, mannose, lactose, complex medium	PLFA	GC/C/IRMS	Complex fractionation patterns varying with substrate and organism	Abraham et al. (1998)
Woodland and grassland soils	Universally labeled starch, xylose, vanillin and litter	PLFA	GC/C/IRMS	Similar microbial groups responsible for degrading simple substrates in woodland and grassland soils but different communities degraded complex substrates	Waldrop & Firestone (2004)
Estuarine sediments	[U-^{13}C]acetate	PLFA	GC/C/IRMS	Acetate consumed by sulphate reducing bacteria similar to Gram +ve *Desulfotomaculum acetoxidans* and not by a population of Gram -ve *Desulfobacter* spp.	Boschker et al. (1998)
Experimental soils	[1-^{13}C]sodium acetate	PLFA, neutral lipids, glycolipids	GC/MS SIM	Incorporation greatest into PLFA; bacterial growth limited at low pH but occurred at pH 7 and 8	Arao (1999)
Rhizosphere rice paddy soil	$^{13}CO_2$	PLFA	GC/C/IRMS	Microbial populations in rice soil differ in their response to plant photosynthate input	Lu et al. (2004)
Rhizosphere grassland soil	$^{13}CO_2$	PLFA	GC/C/IRMS	^{13}C-labeling showed fundamental differences in the way rhizodeposition was cycled through microbial community during different stages of plant development	Butler et al. (2003)

Rhizosphere grassland soil	$^{13}CO_2$	PLFA	GC/C/IRMS	Fungal and Gram -ve bacterial PLFAs showed most ^{13}C enrichment. Liming did not affect assimilation or turnover rates of ^{13}C-labeled C	Treonis et al. (2004)
Sediments from petroleum contaminated aquifer	[*methyl*-^{13}C]toluene	PLFA	GC/C/IRMS	PLFAs resemble those of PHC-degrading *Azoarcus* spp.	Pelz et al. (2001)
Petroleum contaminated groundwater	[2-^{13}C]acetate	PLFA (FISH)	GC/C/IRMS	Field-scale application of acetate to investigate carbon assimilation and mineralization	Pombo et al. (2002)
Petroleum contaminated aquifer water and sediment	[2-^{13}C]acetate	PLFA (FISH)	GC/C/IRMS	Main sulfate reducing bacteria degrading acetate in water *Desulfotomaculum acetoxidans* and *Desulfobacter* in sediment	Pombo et al. (2005)
Antarctic soil bacteria	^{13}C-labeled grass	Ergosterol, PLFA, NLFA	GC/MS SIM	Incorporation of ^{13}C increased over incubation period which was not seen in PLFA and NLFA fractions	Malosso et al. (2004)
Soil	Ring labeled [^{13}C]toluene and [U-^{13}C]glucose	PLFA	GC/C/IRMS	Specific labeling patterns for microbial PLFAs from ^{13}C-toluene incubation contrasting universal labeling of PLFAs from incubating with ^{13}C-glucose	Hanson et al. (1999)
Batch culture	[U-^{13}C]toluene	PLFA	GC/C/IRMS	Quantified carbon flow along substrate–bacteria–protist food chain	Mauclaire et al. (2003)
Soil	$^{13}CH_4$	Hopanoids	GC/C/IRMS	Specific bacteriohopanoids labeled	Crossman et al. (2001)
Upland soils	$^{13}CH_4$	PLFA, DGGE	GC/C/IRMS	Different methanotrophs are present in different soils that oxidize atmospheric methane	Knief et al. (2003)
Landfill cover soils	$^{13}CH_4$	PLFA	GC/C/IRMS	Changes in methanotrophic community from type I to type II with depth	Crossman et al. (2004)

(Continued)

Table 14.3 *Continued*

Environment	^{13}C-labeled substrate	Biomarker	Detection method	Conclusion	Reference
Soil	$^{13}CH_4$	PLFA, hopanoids	GC/C/IRMS	Novel population of methane-oxidizing bacteria related to type II methanotrophs, *Methylocapsa* and *Methylocella*	Crossman et al. (2005)
Sediment/soil	$^{13}CH_4$	PLFA	GC/C/IRMS	Shift in the composition of the methane oxidizing bacterial community in the sediments/soils treated with ammonium	Nold et al. (1999); Crossman et al. (2006)
Peat bog	$^{13}CH_4$	Hopanoids, sterols (FISH)	GC/C/IRMS	Methanotrophic bacteria associated with *Sphagnum* mosses providing CO_2 for photosynthesis	Raghoebarsing et al. (2005)
Sediment	Biogenic CH_4	Hopanoids	GC/C/IRMS	Hopanoids exhibit depleted $\delta^{13}C$ values as a result of their production by methanotrophic bacteria	Freeman et al. (1990)
Mussel gill tissue	Biogenic CH_4	Methanotroph biomarkers	GC/C/IRMS	Detection of methanotrophic symbionts associated with the mussel	Jahnke et al. (1995)
Sediment	Biogenic CH_4	Archaeal lipids	GC/C/IRMS	Archaeal lipids are depleted in ^{13}C indicating that archaea are involved in methane consumption. Abundances differ between sites suggesting multiple archaeal species	Pancost et al. (2001)
Anaerobic oxidation of methane (AOM)	$^{13}CH_4$	PLFA, archaeal lipids	GC/C/IRMS	^{13}C uptake into specific lipids indicate phylogenetically distinct microbes participate in AOM	Blumenberg et al. (2005)

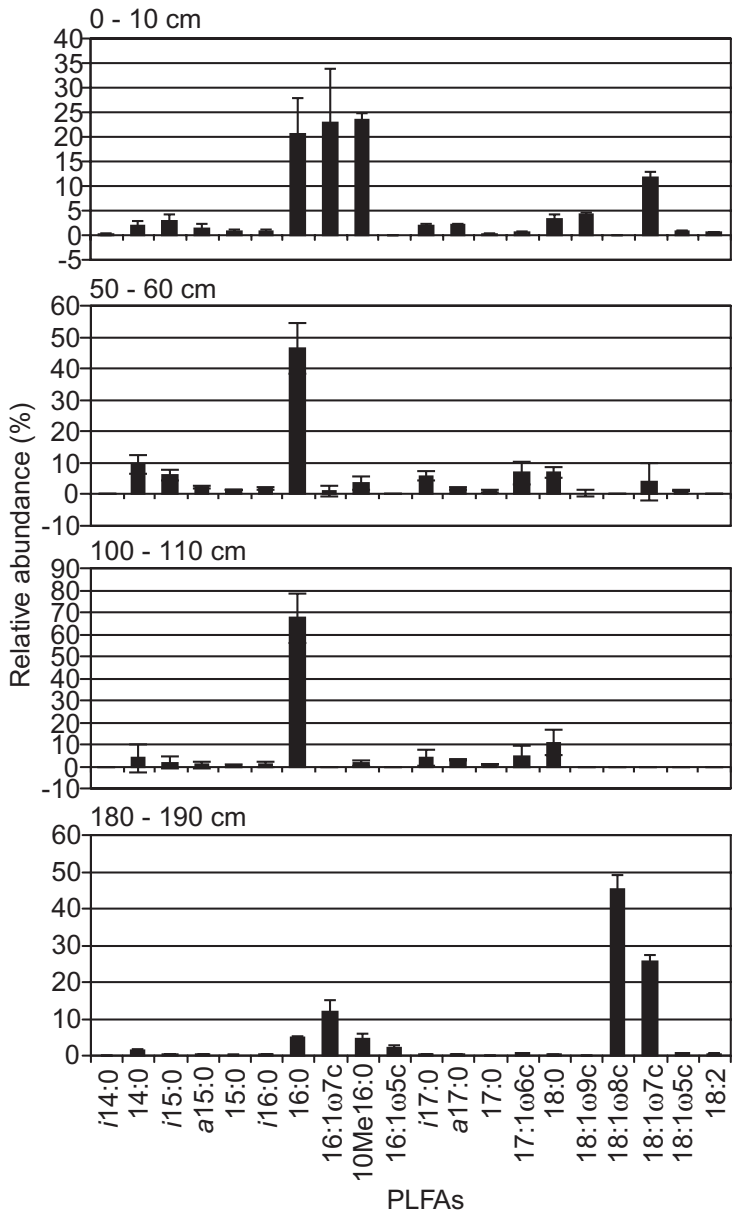


Figure 14.7 Relative abundances of ^{13}C-labeled PLFAs extracted from four sections of the profile of a landfill cap following incubation with 10,000 ppm methane containing 1% $^{13}CH_4$. (Adapted from Crossman et al. 2004.)

bacteria, while those of the propionate consumers did not resemble any known strain.

Use of compound-specific stable isotopes to investigate soil organic matter cycling, pollution, and biodegradation

Table 14.4 summarizes a number of key environmental applications that have successfully utilized a compound-specific stable isotope approach. The three main environmental areas are considered in turn below.

Soil organic matter cycling

The majority of investigations into soil organic matter preservation and diagenesis have focused on the ^{13}C content of bulk fractions with surprisingly few adopting molecular approaches (Lichtfouse 1995, 1998; Lichtfouse et al. 1994, 1995; Huang et al. 1996). Lichtfouse (1995) used the difference in carbon isotope composition of C3 and C4 plants to trace the increasing incorporation, with time, of the C_{29} long chain *n*-alkane from maize epicuticular wax into a soil that had previously been cultivated with C3 plants. Huang et al. (1996) determined the isotopic composition of *n*-alkanes and triterpenoids through the profile of three different soils, finding that, with increasing depth, the *n*-alkanes became progressively more enriched in ^{13}C. They interpreted this as an addition of enriched alkanes from microbial sources. Additionally, they also reported hopanoids enriched by 4–5‰ compared with plant *n*-alkanes, leading to the conclusion that these lipids were biosynthesized from carbohydrates or proteins from the plant. Lichtfouse (1998) observed that long-chain fatty acids, *n*-alkanes, and alcohols in soils exhibit carbon isotope values consistent with their derivation from C3 plant waxes, however, shorter chain fatty acids (C_{16}–C_{18}) have higher $\delta^{13}C$ values indicating that they are most likely biosynthesized by the microbial biomass in the soil. This is consistent with the results from a previous study by Lichtfouse et al. (1995) where ^{13}C-labeled glucose was applied to a soil and the extractable fatty acids, *n*-alkanes, and *n*-alkanols were analyzed by GC/C/IRMS and the short-chain fatty acids exhibited significant enrichment in ^{13}C as a result of their biosynthesis by microbes from the labeled glucose.

Pollution studies involving compound-specific stable isotope analyses

Source apportionment

Different manufacturing pathways and differing raw materials result in the production of materials with distinct isotopic signatures for their site of origin.

Table 14.4 Environmental studies utilizing compound-specific stable isotope approaches.

Environment	^{13}C-labeled substrate	Biomarker	Detection method	Conclusion	Reference
Groundwater	MTBE fractionation	MTBE	GC/C/IRMS	Biodegradation of MTBE causes enrichment in ^{13}C more than physical processes	Hunkeler et al. (2001)
Coastal Lagoon	Biogenic CH_4	PAHs	GC/C/IRMS	PAHs derive from industrial plant utilizing methane as a carbon source rather than a petrogenic source	McRae et al. (2000)
River	PCBs of known isotopic composition	PCBs in duck tissue, duck liver, duck egg and grass carp	GC/C/IRMS	Isotopic patterns provide information on the relative metabolic stability and degradation processes of various PCB congeners in different animals	Yanik et al. (2003)
Soil	Coal derived PAHs	PAHs	GC/C/IRMS	Coal derived PAHs can be resolved from those derived from petroleum in contaminated land	Sun et al. (2003)
Sediments	Fuel *n*-alkanes	*n*-alkanes	GC/C/IRMS	Isotopic values of *n*-alkanes from contaminated sediment correlate with those shipping bunker fuel *n*-alkanes	Rogers & Savard (1999)
Soil	Fuel derived PAHs	PAHs	GC/C/IRMS	The resolution of PAHs derived from automobile exhaust, tyre abrasion and tailpipe soot is attempted, however differences were too small to be conclusive	Glaser et al. (2005)
Groundwater – microcosms and field site	MTBE isotopic fractionation	MTBE	GC/C/IRMS	Anaerobic biodegradation of MTBE causes extensive fractionation of hydrogen and carbon isotopes	Kuder et al. (2005)
Tropical and temperate soils and termite nests	PAHs	PAHs	GC/C/IRMS	Tropical soil PAHs exhibit significant inputs of biogenic PAHs compared with temperate soil PAHs that are mainly from pyrolytic sources	Wilcke et al. (2002)
Laboratory microcosm and aquifer	BTEX and PAH fractionation	BTEX and PAHs	GC/C/IRMS	Significant biodegradation of toluene and o-xylene occurs along the groundwater flow path. Certain aromatic hydrocarbons do not exhibit isotope fractionation with microbial degradation, e.g., indane and indene	Richnow et al. (2003a)

Table 14.4 *Continued*

Environment	^{13}C-labeled substrate	Biomarker	Detection method	Conclusion	Reference
Landfill leachate plume	BTEX fractionation	BTEX	GC/C/IRMS	Isotope fractionation similar to previous laboratory based study indicated that biodegradation of ethyl benzene and m/p-xylene had occurred. Other compounds were at such low concentrations that isotope values could not be recorded accurately	Richnow et al. (2003b)
Oil spill, tar balls and oil-coated feathers	*n*-alkane and PAHs from Erika oil spill	*n*-alkanes and PAHs	GC/C/IRMS	Oil on bird feathers is correlated by biomarkers and isotope ratios with the oil spilled by Erika tanker. Tar balls derive from elsewhere	Mazeas & Budzinski (2002)
Soil	Fatty acids, alcohols and *n*-alkanes	Fatty acids, alcohols and alkanes	GC/C/IRMS	Long chain fatty acids, *n*-alkanes and *n*-alcohols are derived from C_3 plant sources, however, C_{14}-C_{18} fatty acids have a higher content of ^{13}C consistent with biosynthesis by microbial or fungal biomass	Lichtfouse et al. (1995)
Soil	Maize C_{31} *n*-alkane	C_{31} *n*-alkane	GC/C/IRMS	Enrichment in ^{13}C of soil *n*-alkane over time as maize epicuticular wax *n*-alkane is incorporated into the soil	Lichtfouse (1995)
Soil	Alkanes and triterpenoids	*n*-alkanes and triterpenoids	GC/C/IRMS	More enriched δ^{13}C values interpreted as inputs from microbes utilizing plant protein or carbohydrate	Huang et al. (1996)
Soil	^{13}C-labeled glucose	Fatty acids	GC/C/IRMS	C_{14}, C_{16} and C_{18} fatty acids became enriched in ^{13}C as a result of their biosynthesis by microbes, however, long-chain fatty acids, *n*-alkanes and alcohols derived from plants and therefore were not labeled	Lichtfouse (1998)
Soil	C_4 Plant	*n*-alkanes	GC/C/IRMS	Isotope values of soil *n*-alkanes increased with time of maize cultivation as a result of maize carbon integration into soil organic matter	Lichtfouse et al. (1994)

These distinct signatures can be used in conjunction with molecular analysis in order to establish the source of particular compounds. This information is useful for determining how the substance entered the environment, in particular, when identifying responsible parties for further examination of practices. Furthermore it is possible to assess whether these compounds are a result of contamination, produced naturally, or are degradation products of a contaminating agent. Prior to the use of isotopic analysis the majority of this type of work rested upon biomarker analysis, whereby particular sources are matched with residues by their molecular compositions: the robustness of this technique is improved considerably by combining it with GC/C/IRMS analysis.

The isotopic composition of certain biomarker components of pollutants can be used to determine the source of these pollutants in various environmental samples. The majority of these investigations have focused on the source of *n*-alkanes or polyaromatic hydrocarbons (PAHs) deriving from oil pollution. Rogers & Savard (1999) correlated the isotopic composition of *n*-alkanes deriving from sediment samples with those of a shipping bunker fuel illustrating that a combination of isotopic and biomarker analyses can be used to determine the source of the sedimentary pollution. This technique was also employed by Mazeas & Budzinski (2002) who sampled oil residues and oiled bird feathers from the coast of France following an oil spillage. By combining PAH and *n*-alkane compositional and isotopic analyses they were able to determine that the bird feathers were contaminated with oil from the slick, however, many of the oil residues found likely derived from other oil tankers cleaning their tanks. McRae et al. (2000) observed isotopically light PAHs in lagoon sediments which they concluded were emitted by a nearby plant that used biogenic methane as a carbon source rather than petrogenic sources. In addition to pollution of marine environments, studies have also focused on soils (Wilcke et al. 2002; Sun et al. 2003; Glaser et al. 2005). Sun et al. (2003) were able to resolve coal-derived PAHs from those derived from petroleum sources in contaminated land as a result of their differing isotopic compositions. Wilcke et al. (2002) used the isotopic signature of perylene to show that in tropical environments recent biological sources of PAHs from termites were present, whereas, temperate soil PAHs were primarily formed from pyrolytic sources.

Biodegradation

A further use of the isotopic compositions of pollutant residues is in the investigation of the extent and processes of their biodegradation. A more in depth overview of the majority of studies of biodegradation of organic contaminants investigated by compound-specific isotope analysis is given by Schmidt et al. (2004). In general, as compounds are biologically degraded

their isotopic compositions are changed as a result of the organism preferentially removing the lighter isotope to leave the residual compound isotopically enriched compared with its initial composition. This is illustrated by Hunkeler et al. (2001) who showed that as methyl *tert*-butyl ether (MTBE) was biodegraded an isotope fractionation occurred leaving the residual MTBE isotopically heavier, however, when phase partitioning occurred the fractionation was much smaller. This was confirmed by studies of Kuder et al. (2005) who determined $\delta^{13}C$ and δD values of MTBE in an anaerobic enrichment culture. They reported extensive fractionation of both isotopes as a result of anaerobic biodegradation and were able to detect biodegradation in groundwater samples at nine gasoline spill-sites, in some cases exceeding 90% of the original mass. In addition to MTBE biodegradation, Richnow et al. (2003a, 2003b) have used the isotopic composition of benzene, toluene, ethylbenzene, and xylenes (BTEX) to investigate their biodegradation. They performed a laboratory based study of biodegradation to obtain the fractionation factors for a variety of BTEX and applied this factor in the field to observe biodegradation of BTEX along a groundwater flow path and a landfill leachate plume. This was found to correlate with the change in concentration along the gradient. However, they found that not all BTEX tested produced an observable isotope fractionation with biodegradation.

Application of compound-specific stable isotope analysis for trophic studies of invertebrates

Compound-specific stable isotopic techniques have proven to be valuable in studies involving C flow and trophic studies in aquatic and terrestrial systems, especially when direct observations of feeding are difficult (Rieley et al. 1999; Ziegler & Fogel, 2003; Chamberlain et al. 2004; McCarthy et al. 2004). In particular, fatty acid compositions and their associated $\delta^{13}C$ values have been utilized effectively in confirming trophic interactions, since certain fatty acids are conserved during trophic transfer, thus can be used as biomarkers. Studies using this approach have found evidence of chemosynthetic mussels and tube worms incorporating the fatty acids of symbiotic bacteria (MacAvoy et al. 2002). The fatty acid $\delta^{13}C$ values determined for the mussels (−45.4‰ to −39.6‰ or −78.8‰ to −68.4‰), reflected the methane source utilized by their symbionts, whereas the tube worm fatty acid $\delta^{13}C$ values (−29.3‰ to −18.0‰) reflected the local dissolved inorganic carbon (DIC) pools due to the tube worm symbionts utilizing the DIC as their C source. The results demonstrated that chemosynthetically produced essential and precursor fatty acids isolated from host tissue retain the $\delta^{13}C$ values of the synthesizing bacteria. Consequently, the C source used by the symbionts can be identified and the essential fatty acid $\delta^{13}C$ values can be used to trace the fatty acids transferred between organisms. Similarly, Pond et al. (1998) determined the stable carbon isotope composition of fatty acids in chemosynthetic mussels and

confirmed that thio- and methanotrophic bacterial endosymbionts were equally important in the nutrition of the mussel.

The trophic behavior of shrimps has been studied by adopting similar compound-specific methods (Pond et al. 1997, 2000; Rieley et al. 1999; Pakhomov et al. 2004). Rieley et al. (1999) investigated the trophic ecology of the alvinocaridid shrimp *Rimicaris exoculata* by determining the molecular composition and $\delta^{13}C$ values of its PLFAs and sterols. The values were compared with the three main sources of dietary C available, and the results suggested that the predominant source of dietary C for the shrimp was from the epibiotic bacteria living within their carapaces, and that the shrimp cholesterol was likely to be derived from the oceanic photic zone.

Nitrogen stable isotope signatures are also important in trophic studies, and can be used as an indicator of trophic level (DeNiro & Epstein 1981). The $\delta^{15}N$ values of some amino acids becomes heavily enriched from food source to consumer, while others exhibit little or no change and thus can provide information regarding the source of N (McClelland & Montoya, 2002). Pakhomov et al. (2004) determined the $\delta^{15}N$ values of amino acids of the bottom-dwelling caridean shrimp *Nauticaris marionis* to study its spatial and temporal variability. The amino acid $\delta^{15}N$ values suggested that the *N. marionis* from the inter-island realm were second-order carnivores, whereas the shrimps at the nearshore realm were first-order carnivores. This was not revealed in bulk $\delta^{15}N$ values, which suggested that the trophic levels did not differ, thus highlighting the advantage of compound-specific stable isotope analyses.

Carbon isotope analysis of PLFAs can be used to delineate algal and bacterial material from other organic matter in estuaries, and to trace the differential dynamics and isotope fractionation of green algae and diatoms along the estuarine gradient. Boschker et al. (2005) investigated the planktonic community structure along the Scheldt estuary using $\delta^{13}C$ values of PLFAs. This study revealed that the $\delta^{13}C$ values of the diatom fatty acid biomarker, 20:5ω3, were primarily related to DIC $\delta^{13}C$ values, and that green algae and diatom markers use a different inorganic C source or carbon-fixation metabolism, due to the difference in the $\delta^{13}C$ values of the green algal fatty acid biomarker, 18:3ω3, and the diatom fatty acid biomarker, 20:5ω3. The isotopic composition of the three bacterial PLFAs, i15:0, a15:0, and 18:1ω7c, closely followed particulate organic carbon (POC) $\delta^{13}C$ values, indicating that POC was the main source for bacterial growth. At the marine end of the estuary, algal PLFAs were similar in $\delta^{13}C$ values to bacterial PLFAs and POC, suggesting that local production by phytoplankton may be an important source for bacterial growth in that region of the estuary. However, in the upper estuary algal PLFAs were more depleted than bacterial PLFAs, and the isotope signatures suggest that the bacterial C substrate was primarily of terrestrial or anthropogenic sewage origin.

Combining compound-specific stable isotope analysis with ^{13}C-labeling provides further opportunities for monitoring the presence and growth of

individual populations in planktonic and benthic communities, and to track C and fatty acid transfer along the food chain to primary and secondary consumers (Pel et al. 2004, Middelburg et al. 2000). Such studies have revealed rapid and significant transfer of C from benthic algae to bacteria and nematodes, with maximum labeling after 1 day (Middelburg et al. 2000), and that zooplankton, previously thought to be predaceous, grazed mainly on algae (Pel et al. 2004).

Compound-specific stable isotope analysis can also provide new insights into food web analyses; $\delta^{13}C$ values of individual compounds offer the advantage of providing additional isotopic information regarding the assimilation and biosynthesis of specific compounds from an organism's diet, which also assist in tracing the origins of nutrients necessary for biomass production. However, stable isotope composition can change between diet and consumer due to differential digestion or fractionation during assimilation and metabolic processes. Metabolic fractionation can also result in $\delta^{13}C$ values of different tissues varying substantially within a consumer (Hobson & Clark 1992). Furthermore, there are differences in the isotopic composition of the major biochemical classes, e.g. lipids usually possess more depleted $\delta^{13}C$ values than other major compound classes and the whole organism, due to enzymatic discrimination against ^{13}C during lipid biosynthesis (DeNiro & Epstein 1977). Hammer et al. (1998) determined the $\delta^{13}C$ values of individual fatty acids extracted from subcutaneous fat tissues of redhead ducks, *Aythya americana*, and the roots and rhizomes of the seagrass, *Haloduke wrightii*, assumed to be the main dietary source for at least a month. The $\delta^{13}C$ values of the fatty acids in the ducks were more positive than the identical fatty acids in the seagrass. The $\delta^{13}C$ values of the duck fatty acids were generally closer to the $\delta^{13}C$ value of the whole tissue of the seagrass roots and rhizomes. This strongly indicates that the ducks do not directly incorporate the fatty acids of their diet into their fat tissues, at least not to a large extent, and that the fatty acids are biosynthesized from precursors with more positive $\delta^{13}C$ values, such as carbohydrates and/or proteins. However, there may also be isotopic fractionations associated with the biosynthesis and catabolism of these compounds.

Carbon isotope compositions of individual amino acids can also be used to define isotope fractionations related to specific metabolic processes. Fantle et al. (1999) demonstrated that the experimentally cultured juvenile blue crab, *Callinectes sapidus*, fractionates the C isotopes of nonessential amino acids to a greater extent than essential amino acids, implying different metabolic pathways. Depletions in $\delta^{13}C$ values in the detritus fed crab also revealed that crabs feeding on detritus selectively assimilated an isotopically distinct pool of C, rather than the bulk detritus. In addition, the $\delta^{13}C$ values of the individual amino acids from crabs collected from the bay and marsh of a natural estuarine system, indicated that crabs in the bay and marsh utilize different sources of C, but shared a protein-rich dietary component, most likely

plankton, which contributed significantly to their essential amino acid requirement. The $\delta^{13}C$ values of amino acids have further been employed to study the dietary sources of egg amino acids in nectar-feeding insects (O'Brien et al. 2002, 2005). The insects were raised on isotopically contrasting diets (C3 and C4) and the egg amino acid $\delta^{13}C$ values determined. The results showed that the adult diet had a highly significant effect on nonessential amino acid $\delta^{13}C$ values but no adult dietary C was incorporated into any of the essential amino acids, therefore essential egg amino acids originate entirely from the larval diet.

The potential of compound-specific stable isotope analysis as a powerful tool for the study of C dynamics and trophic interactions in soil ecosystems has only recently been explored. Chamberlain et al. (2004) examined the implications for the use of compound-specific carbon isotope analysis in animal dietary studies by investigating the rate of incorporation of dietary C into collembolan lipids by switching Collembola (*Folsomia candida* and *Proisotoma minuta*) from C3 to C4 diets, and then determined the $\delta^{13}C$ values of fatty acids over a period of 39 days (Chamberlain et al. 2006a). The $\delta^{13}C$ values of the collembolan lipids were often different to those of the same components in the diet, suggesting that fractionation or partitioning occurs during digestion, assimilation, and biosynthesis within the Collembola. The rates of change of $\delta^{13}C$ values differed among compounds, and half-lives ranged from 29 min to 14 days, some of which were related to the abundance of the component in the diet. This highlights the importance of determining potential sources of isotopic fractionation so that meaningful interpretations can be achieved.

Trophic preferences of soil invertebrates are mainly conducted by gut content analysis, or through visual observations, which can be labor intensive and often unreliable. Chamberlain et al. (2006b) have recently applied compound-specific stable carbon isotope analysis to determine the trophic preference of two species of Collembola, *F. candida* and *P. minuta*. The diets were isotopically distinct to facilitate the determination of collembolan food sources. Furthermore, any isotopic fractionation or partitioning occurring would be small relative to the stable C isotope signature of C derived from the C3 and C4 diets. The results showed that the collembolan fatty acid $\delta^{13}C$ values increased over 21 days, reflecting those of the nematode diet (C3 signature), suggesting a strong feeding preference for the nematodes over the fungi, even though the species of Collembola are classically considered to be mycophagous. Figure 14.8 shows the $\delta^{13}C$ values of fatty acids 16:0 vs. 18:0 and 16:1ω7 vs. 18:2ω6, for each of the Collembola and the offered diets. The $\delta^{13}C$ values of 16:0 and 18:0 in *F. candida* (Figure 14.8a) lie close to those of the nematode, but for *P. minuta* they appear to be intermediate between both diets, implying that *P. minuta* consumed both fungi and nematodes. However, the $\delta^{13}C$ values of 16:1ω7 and 18:2ω6 (Figure 14.8b) for both *F. candida* and *P. minuta* plot close to the $\delta^{13}C$ values of *P. redivivus*, consistent with this diet being the only

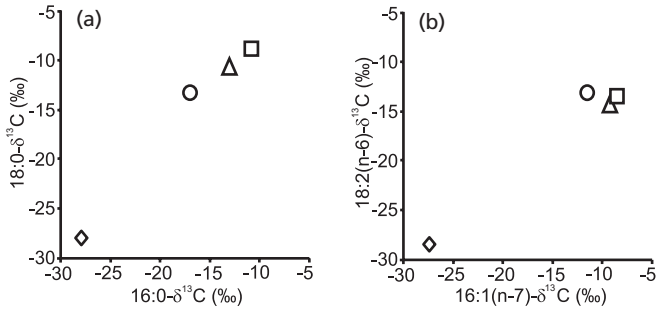


Figure 14.8 $\delta^{13}C$ values of selected fatty acids derived from diets (*C. cladosporioides* and *P. redivivus*), and consumers (*F. candida* and *P. minuta*) after 21 days of the preference test. (a) 16:0 vs. 18:0; (b) 16:1ω7) vs. 18:2ω6. Errors associated with the $\delta^{13}C$ values are smaller than the symbols. ◇ *C. cladosporioides*; □ *P. redivivus*; △ *F. candida*; ○ *P. minuta*. (Adapted from Chamberlain et al. 2006b).

significant C source consumed by the Collembola. This study demonstrates compound-specific stable isotope analysis as a powerful tool for determining trophic preferences of Collembola, and potentially many other classes of soil invertebrates, offering significant improvements over traditional methods.

Pulse labeling of above-ground vegetation with $^{13}CO_2$ *in situ* has recently been combined with compound-specific analysis to determine C assimilation by individual enchytraeid worms (Black et al., unpublished results). Cholesterol was extracted from the individual enchytraeids and $\delta^{13}C$ values determined by GC/C/IRMS. Rhizosphere C assimilation was demonstrated by significant changes in the cholesterol $\delta^{13}C$ values. Results showed that no significant differences were observed in the $\delta^{13}C$ values of cholesterol in the two dominant genera, but variation in the intra-population indicate that the C may be assimilated in different ways, and large variations in the $\delta^{13}C$ values suggest that individual worms may have a range of food sources. In addition, Derrien et al. (2003, 2004) applied ^{13}C-labeling and compound-specific stable isotope techniques to investigate the nature and dynamics of individual carbohydrates in the rhizosphere. They demonstrated that in wheat, neutral sugars were the dominant components released into the soil by living roots, and that polymerized glucose was the most abundant compound of the rhizodeposits. The combined use of ^{13}C-labeling and compound-specific stable isotope analysis clearly shows potential for providing critical information regarding C dynamics and trophic interactions in soil environments.

Conclusions

Major impetus was given to the field of compound-specific stable isotope analysis through the development of the GC/C/IRMS technique and the past

decade has seen an explosion in the number and range of applications of the approach.

Application of compound-specific stable isotope approaches in the field of organic geochemistry has become routine and has led to many advances in our understanding of past environments. The major advantage in using compound-specific approaches in paleoecological reconstructions stems from the ability to match structures to isotope values to give greater specificity to biomarker studies, and this provides enhanced insights into sedimentary processes and biogeochemical cycles. Adoption of the approach into other research areas has been surprisingly slow, possibly due to unfamiliarity of workers with the molecular level analytical approaches that are implicit in organic geochemistry. Familiarity with the separation and characterization methods underpins all applications of compound-specific stable isotope analyses and is essential to the success of the approach.

The potential for the further application of compound-specific approaches to improve our understanding of biogeochemical cycles in contemporary environments is considerable indeed. The accumulating examples of studies involving stable isotopes as tracers, essentially replacing radioactive tracers in some investigations, are testimony to the possibilities offered by the approach. As laboratories acquire the necessary instrumentation the next few years is set to see a major expansion in the application of compound-specific approaches to studies of microbes in the environment, combined with genetic approaches. Such approaches are proving to be especially powerful in providing new insights, especially into unculturable microbes, since ^{13}C-labeled tracers can be introduced with minimal disturbance into natural systems. The major challenges lie with the design of imaginative and robust experiments.

Likewise, such approaches have the potential to probe the activities of micro-invertebrates in environments where possibilities of visual observations are limited or impossible. The possibilities in this area are enhanced by the development of techniques for the stable carbon isotope analysis of individual organisms (e.g. Evans et al. 2003), which have the capacity to reveal the behavior of a species at the level of the individual rather than the population. Unlike radiolabeled tracers no ethical problems exist in the application of stable isotope tracers in natural systems, thereby allowing biochemical pathways and ecosystem processes to be studied in a diverse range of natural and anthropogenically altered environments. The introduction of novel stable isotope tracer delivery systems is helping to increase the range of applications still further (Ostle et al. 2000).

Considerable promise is offered in the field of archaeology for the further application of compound-specific stable isotope approaches due to the survival of biochemical components in association with archaeological materials, such as skeletal remains and pottery. This has proved to be an area where stable isotope approaches focus mainly on mammals, including both humans

and domesticated species. In the field of paleodietary reconstruction the compound-specific approaches have clear potential and complement the bulk stable isotope determinations that have been extensively used for around three decades. Recent work has shown that otherwise inaccessible information becomes available via compound-specific studies of the amino acids that comprise collagen (Corr et al. 2005). Likewise, new possibilities exist for using largely untapped sources of light stable isotope information contained in such compounds as cholesterol, which reflect different aspects of diet and environment (Jim et al. 2004). A surprisingly neglected area appears to be the application of compound-specific stable isotope approaches in our understanding of avian behavior (Hammer et al. 1998) despite the intense interest in the use of stable isotopes in this field.

While instrumentation for the stable isotope analysis of other light isotopes is commercially available surprisingly few publications have emerged for compound-specific studies of nitrogen (e.g. Petzke et al. 2005), deuterium (e.g. Xie et al. 2000) and oxygen (Hener et al. 1998). The next few years will undoubtedly see an expansion in the range of applications of these techniques in a variety of fields.

A recent major development has been the first reported applications of an operational high-performance liquid chromatograph (HPLC)–IRMS. The new HPLC–IRMS method appears to offer significant advantage over the GC/C/IRMS for certain applications. First, it extends the range of compounds that can be investigated. For example, in the case of proteins or peptides they can be studied intact, or if $\delta^{13}C$ values are required for their component amino acids then only purification and hydrolysis is required, rather than derivatization. Analytes separated by aqueous based HPLC are quantitatively converted to CO_2 in a reactor containing sodium peroxodisulphate and phosphoric acid. Preliminary analyses have shown that amino acids can be analyzed directly on such HPLC-IRMS systems to provide accurate $\delta^{13}C$ values (Krummen et al. 2004). Analytical precisions reported for a range of amino acids are ca. ±0.2–0.3‰ for 100–300 ng amino acid injected. Clearly, there is great potential for the application of this technique in a wide range of fields adding yet further scope to the application of compound-specific stable isotope analyses in ecological and paleoecological studies.

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